University of Alberta

Predicting the Biologic Behavior of Gastric Cancer: An Application of Gene-Expression and Tissue Array Techniques

By

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ABSTRACT

The objectives of this study were to determine the generalizability of independent clinicopathological predictors of survival in patients with gastric cancer, to incorporate selected protein markers into a multivariate analysis using multi-tumor tissue arrays and to explore gene-expression profiles, using a prospectively gathered fresh gastric cancer tissue.

A multivariate model was constructed to examine independent predictors of survival in a population-based cohort residing in Northern Alberta. A gastric cancer multi-tumor tissue array was examined for protein immunoreactivity patterns with respect to lymphovascular invasion (LVI), and incorporated into a multivariate model. Prospectively gathered gastric cancer specimens were collected for tumor banking and DNA microarray analyses.

Multi-tumor tissue arrays are an efficient method of incorporating marker protein immunoreactivity into multivariate models, providing important information about the biologic behavior of gastric cancer. DNA microarray analyses are a feasible method of examining the biologic behavior in cancer, and improving our understanding of gastric cancer.

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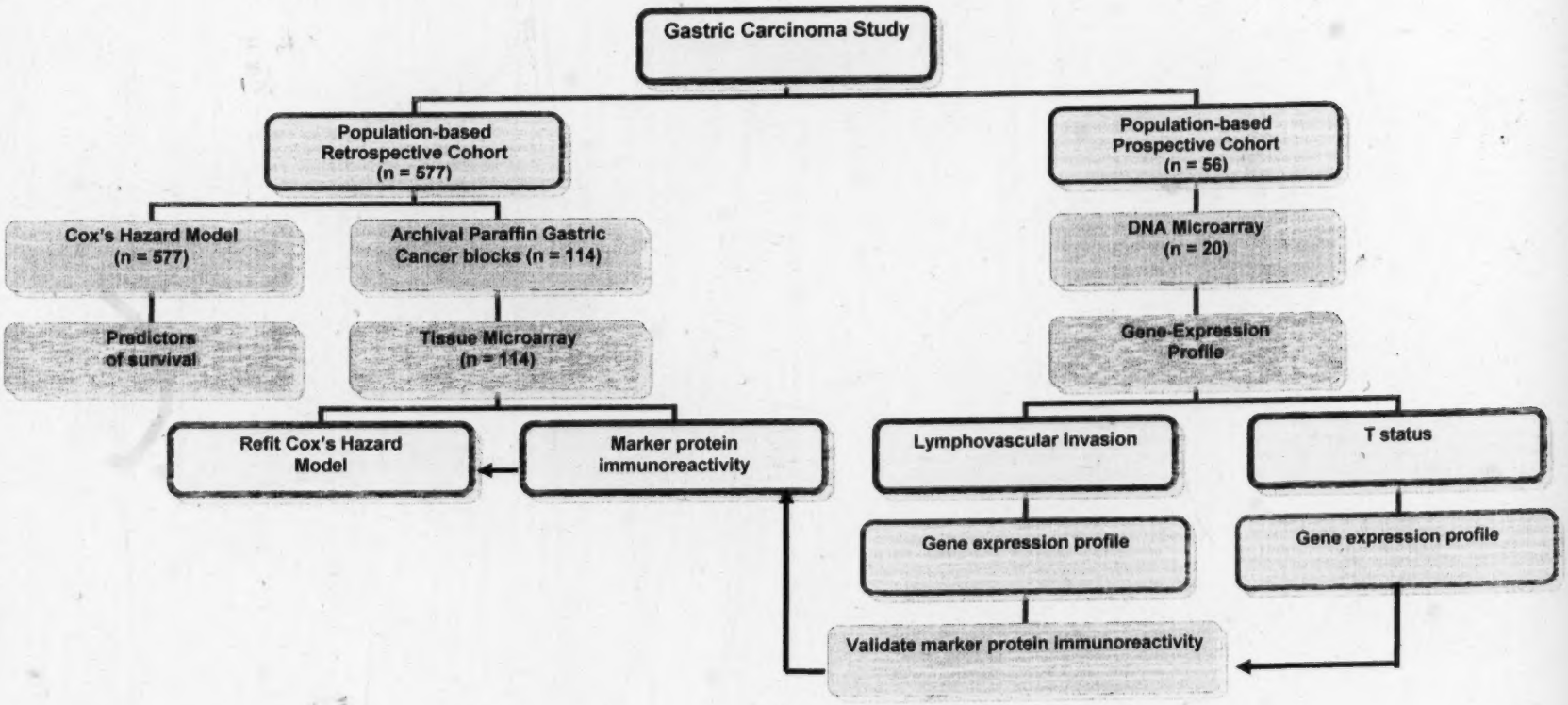
Predicting the Biologic Behavior of Gastric Cancer: An Application of Gene-Expression and Tissue Array Techniques: a schematic representation


Gastric Carcinoma Study
Population-based Retrospective Cohort (n = 577)
Population-based Prospective Cohort (n = 56)
Cox's Hazard Model (n = 577)
Archival Paraffin Gastric Cancer blocks (n = 114)
DNA Microarray (n = 20)
Predictors of survival
Tissue Microarray (n = 114)
Gene-Expression Profile
Refit Cox's Hazard Model
Marker protein immunoreactivity
Lymphovascular Invasion
T status
Gene expression profile
Gene expression profile
Validate marker protein immunoreactivity

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Chapter 1

Introduction and Background

1.1 Introduction

Gastric cancer is the second most common cancer worldwide, with a frequency that varies greatly across different geographic locations.[1] It is a relatively infrequent neoplasm in North America, yet contributes substantially to the burden of cancer deaths.[2-5] In North America, gastric cancer is the third most common gastrointestinal malignancy after colorectal and pancreatic cancer, and the third most lethal neoplasm overall.[4] Despite the decreasing worldwide incidence, gastric cancer accounts for 3-10% of all cancer-related deaths.[6] Although the survival rate for gastric cancer has steadily improved in countries such as Japan, it has not in North America.[3] The substantial mortality associated with gastric cancer has prevailed in spite of technical advances in surgery and the use of adjuvant therapy.

Ninety percent of all tumors of the stomach are malignant, and gastric adenocarcinoma comprises 95% of the total number of malignancies.[7] Curative therapy involves surgical resection, most commonly a total or subtotal gastrectomy, with an accompanying lymphadenectomy. The overall five-year survival of patients with resectable gastric cancer ranges from 10-30%.[8-10]

1.1.1 Epidemiology

Gastric cancer is rare before the age of 40, but its incidence steadily climbs thereafter and peaks in the 7th decade of life.[11] It is estimated that worldwide 876,340 cases of primary gastric cancer were diagnosed, and nearly 650,000 deaths occurred in 2000.[4] In North America, the lifetime probabilities of developing and dying from gastric cancer are 1.5% and 1.0% respectively.[4] Overall, age-standardized mortality rates have decreased in females (9.9 to 4.2/100,000) and males (21.2 to 9.1/100,000) over the past 30 years in Canada.[5] In the United States (US) there are 24,000 new cases and 14,000 deaths annually.[12] In a retrospective study involving more than 50,000 patients treated for primary gastric cancer, Hundahl et al.[13] demonstrated that 65% of gastric cancers in the US present at an advanced stage (T3/T4), with nearly 85% of tumors accompanied by lymph node metastasis at diagnosis. This problem is complicated further by a recurrence rate of 40-65% in patients resected with curative intent.[14] In the absence of formal

screening programs, most patients present with advanced pathologic stage and can expect a median survival of 24 months (20-30% 5-year survival) in tumors resected with curative intent, a median survival of 8.1 months after palliative procedures and a median survival of only 5.4 months for advanced disease without an operation.[15-17]

1.1.2 Risk Factors

Comparative studies between Asian and western countries demonstrate striking differences in the incidence and overall survival of gastric cancer, which suggest ethnic origin as a possible risk factor.[3, 11, 18] Incidence is highest in Japan (>40/100,000), Eastern Asia, South America, and Eastern Europe; whereas Canada (10/100,000), Northern Europe, Africa and the United States have the lowest incidences.[19] The National Cancer Institute, in an examination of ethnicity as a risk factor for gastric cancer identified three groups: those with high (Koreans, Vietnamese, Japanese, Native American, and Hawaiian), intermediate (Latino, Chinese, African-American), and low age-adjusted incidence of gastric cancer (Filipino, Caucasian).[4]

First-generation migrants from high incidence to low incidence countries sustain the risk rate of their native country whereas, subsequent generations acquire the risk rate of their new environment.[11, 20] This suggests the etiologic influence may reside more in environmental than ethnicity factors.[11] Several dietary and behavioral factors have since been examined in detail. In a case-control study, Ramon et al; [21] identified diets rich in salt, smoked or poorly preserved foods, nitrates, nitrites and secondary amines to be associated with an increased risk of gastric cancer. The association is believed to arise from the prolonged excessive consumption of salty or pickled foods which leads to atrophic gastritis and an alteration in the gastric environment with the generation of carcinogenic N-nitroso compounds.[11] In contrast, diets rich in fruits and vegetables may be associated with a reduced risk of cancer. Haung et al.[20] in a retrospective survey of 877 Japanese gastric cancer patients, suggested that frequent intake of raw vegetables and fruit significantly decreased the risk of gastric cancer-related death (HR=0.74, 95% CI: 0.56 – 0.98) through their antioxidant effects. Calcium, vitamin A, and vitamin C have been postulated to exert a protective effect on the gastric mucosa, through the reduced formation of N-nitroso carcinogenic compounds.[11, 20] A case-control study indicated that cigarette smokers have a 2 to 3 times increased risk of proximal gastric cancer.[22] These

results were supported in a study by Haung et al.[20] demonstrated an odds ratio of 2.53 (CI 1.22-5.29) for habitual smokers, and a trend toward significance in patients with habitual alcohol consumption.

Most gastric cancers occur sporadically, while 8-10% has an inherited familial component.[23] Gastric carcinoma occasionally develops in families with germline mutations in p53 (Li-Fraumeni syndrome) and BRCA2.[19] In 1-3% of gastric cancers, germline mutations in the gene encoding the cell adhesion protein E-cadherin leads to an autosomal dominant predisposition to gastric carcinoma, referred to as hereditary diffuse gastric cancer that has a penetrance of approximately 70%. [19, 24-27] Huntsman et al.[24] suggested that identification of the E-cadherin mutation should prompt prophylactic gastrectomy in affected kindreds. Gastric cancer can develop as part of the hereditary non-polyposis colon cancer (HNPCC) syndrome, as well as part of the gastrointestinal polyposis syndromes including familial adenomatous polyposis (FAP) and Peutz-Jeghers syndrome.[19]

An important development in the epidemiology of gastric carcinoma has been the recognition of the association with *Helicobacter pylori* infection.[19] Three independent studies reported a significantly increased risk in subjects who were demonstrated to have had *H. pylori* infection 10 or more years before the cancer diagnosis.[28-30] A follow-up meta-analysis of 42 observational studies carried out by Eslick et al.[31], showed a significant relationship between *H. pylori* and gastric cancer (OR=2.04; CI 1.69-2.45). *H pylori* has subsequently been shown to induce changes in the gastric mucosa and the gastric flora predisposing to the development of carcinoma in humans.[19] Furthermore, *H. pylori* is capable of adhering to the Lewis blood group antigen, and may be an important factor facilitating chronic infection and the subsequent increased cancer risk observed in patients with blood group A phenotype.[19]

Other factors associated with an increased risk of gastric cancer include chronic atrophic gastritis (eg., pernicious anemia, toxic and dietary agents, previous gastric surgery with bile reflux), hypertrophic gastropathy (Menetrier's disease), gastric polyps, low socioeconomic status, and obesity.[11, 19]

1.2 Gastric Adenocarcinoma: Clinical Considerations

1.2.1 Case Definition/Description

The diagnosis of gastric cancer requires histopathologic assessment of tissue, or cytological assessment of gastric brushing/washes. Several classification systems have been proposed to aid the description of gastric cancer either via macroscopic features (Borrmann) or on the basis of microscopic configuration (Ming, Carniero, and Goseki).[19, 32] The two most commonly used are the Lauren and WHO systems.[19]

The Lauren classification divides gastric cancer into two major histologic types: intestinal and diffuse.[11, 33, 34] This system describes tumors on the basis of microscopic configuration and growth pattern.[11] Diffuse-type cancers have non-cohesive tumor cells diffusely infiltrating the stroma of the stomach and often exhibit deep infiltration of the stomach wall with little or no gland formation.[19, 32] Diffuse tumors may exhibit pronounced desmoplasia and associated inflammation with relative sparing of the overlying mucosa.[32] In comparison to intestinal-type gastric cancers, diffuse-type gastric cancers are less related to environmental influences, have increased in relative incidence, occur more often in young patients and are associated with a worse prognosis.[19] These cancers are not associated with intestinal metaplasia, are not localized to the antrum and may arise out of single-cell mutations within normal gastric glands, as is the case for the newly described hereditary diffuse gastric carcinoma.[23, 24, 35]

Intestinal-type cancers show recognizable gland formation similar in microscopic appearance to colonic mucosa.[11, 19, 32] Glandular formation ranges from well to poorly differentiated tumors which grow in expanding, rather than infiltrative, patterns.[6, 11] Intestinal-type cancers are believed to arise secondary to chronic atrophic gastritis.[11, 19] *H. pylori* and autoimmune gastritis are the commonest etiologic lesions that create an environment conducive to gastric inflammation. If gastritis persists, gastric atrophy occurs followed by intestinal metaplasia, which in turn may lead to dysplasia. Dysplasia can arise in either the native gastric or "intestinalized" gastric epithelium.[19] The term adenoma is applied when dysplastic proliferation produces a macroscopic protruding lesion and is described as tubular, tubulovillous or villous adenoma morphologically.[19] Adenomas tend to occur in the distal stomach, often have a prolonged precancerous phase and an expanding growth pattern.[6, 11, 19] Carcinoma is diagnosed when the tumor invades

into the lamina propria or through the muscularis mucosa.[19] Up to 80% of dysplastic lesions may progress to invasion.

The Lauren classification has proven useful in evaluating the natural history of gastric carcinoma, especially with regard to incidence trends, clinicopathologic correlations and etiologic precursors.[6, 11, 33] Despite the apparent utility of the Lauren classification, the World Health Organization (WHO)[19] has revised the definition of gastric cancer to "malignant epithelial tumors of the gastric mucosa with glandular differentiation." The WHO system assigns grades to adenocarcinoma based on the degree of resemblance to metaplastic intestinal tissue.[6, 19, 32] It categorizes the histologic patterns into five subtypes: adenocarcinoma (intestinal and diffuse), papillary, tubular, mucinous and signet-ring cell.[19, 32]

1.2.2 Clinical Manifestations

Gastric carcinoma often produces no specific symptoms when it is superficial and potentially surgically curable, although up to 50% of patients may have non-specific GI complaints such as dyspepsia.[11] In western countries, even with endoscopic evaluation, gastric cancer is found in only 1-2% of patients suffering with dyspepsia. The lack of early pathogonomic symptoms often delays the diagnosis. Consequently, 80-90% of patients with gastric cancer present with locally advanced or metastatic tumors that have poor rates of resectability.[19] Patients may present with anorexia and weight loss (95%) as well as abdominal pain that is vague and insidious in nature. Nausea, vomiting and early satiety may occur with bulky tumors that obstruct the GI lumen or infiltrative lesions that impair stomach distension.[11] Ulcerated tumors may cause bleeding that manifest as hematemesis, melena or massive upper GI hemorrhage.

Physical examination of early gastric cancer is usually uninformative. Patients with advanced tumors may present with a palpable abdominal mass, cachexia, bowel obstruction, ascites, hepatomegaly and lower extremity edema.[11, 36, 37] Peritoneal seeding may cause involvement of the ovaries (Krukenberg tumor) or pelvic cul-de-sac (Blumer's shelf) detectable on rectal examination.[37] Metastasis may manifest as an enlarged supraclavicular lymph node (Virchow's node), left axillary lymph node (Irish's node) or a periumbilical lymph node (Sister Mary-Joseph's node).[11, 37]

1.2.3 Screening for Gastric Cancer

The goal of mass screening (asymptomatic populations) or surveillance (subjects at risk) is the detection and diagnosis of gastric cancer at an early and therefore potentially curable stage.[19] Mass screening for early detection of gastric cancer is cost-effective and recommended in high incidence regions such as Japan and China, where as many as 50-80% of detected malignancies are early gastric cancers.[19] In North America, there are no formal screening programs. The American Society for Gastrointestinal Endoscopy recommends endoscopic surveillance for high risk individuals (history of gastric adenoma, Familial polyposis syndrome, Peutz-Jegher syndrome, and Menetrier's disease) every 1-2 years.[11] Mass endoscopic and/or radiological screening is not recommended in low incidence areas such as Canada and the United States.[11]

1.2.4 Diagnosis and Staging

Endoscopy is regarded as the most sensitive and specific diagnostic method in patients suspected of harboring gastric cancer.[12] Endoscopy allows direct visualization of tumor location, the extent of mucosal involvement and biopsy (or cytological brushings) for tissue diagnosis.[38] When combined with endoscopy and radiological modalities, endoscopic ultrasound (EUS) can maximize tumor staging by providing information about depth of tumor invasion and assess the extent of peri-gastric lymphadenopathy. Willis et al.[39] suggest that EUS is currently the most valuable diagnostic tool for pre-operative staging of gastric cancer (82% accuracy in assessing the depth of tumor invasion) and for determining tumor resectability. Karpeh et al.[12] suggest the combined use of EUS and laparoscopic staging facilitates patient selection by providing information about tumor depth and peri-gastric lymph node involvement. They do caution however that EUS is less accurate (50-87%) in determining lymph node status.

An upper gastrointestinal barium study (UGI) involves the instillation of liquid barium into the stomach and a combination of 4 techniques: barium-filled evaluation, double-contrast, mucosal relief views and compression views of the stomach.[40] The procedure permits identification of mucosal irregularities. Halvorsen et al.[40] have suggested that, although endoscopy is increasingly becoming the method of choice, the two methods are complementary and have equivalent diagnostic efficacy.

Computer tomography (CT) is the most frequently used modality for staging gastric cancer.[40] CT can detect liver metastases, regional and distant lymphadenopathy and can predict direct invasion of adjacent structures. Kuntz et al.[41] suggested that CT has a sensitivity of 88% for tumor detection. The ability of CT to accurately determine either tumor infiltration (T stage-58%) or peri-gastric lymph node status varied widely (25 – 86%), and was not considered a reliable predictor of disease extent in several studies.[41-43]

Magnetic resonance imaging (MRI) has had limited use in the staging of gastric cancer due primarily to difficulties with motion artifact, cost, time required for examination and lack of an appropriate oral contrast agent.[44, 45] However, in a recent study comparing MRI to CT, Sohn et al.[44] documented advanced gastric cancers were easily detected with both techniques. They showed MRI was slightly better than CT in the T staging (extent of local tumor infiltration) of gastric cancer.[44] Similarly, Kim et al.[46] documented T staging accuracy of MRI was superior to CT (81% vs. 73%, $p< 0.05$). This study suggested MRI was prone to over-staging pathological tumor thickness.[46] Overall T staging accuracy has been reported to be between 73 – 88%.[45] The utility of MRI in N staging (extent of lymph node involvement) has been hindered by the same difficulties encountered with CT staging, where nodal status is judged on the basis of lymph node size. Several studies show the accuracy of MRI nodal staging is inferior to CT staging (65% vs. 73% respectively, $p>0.05$), with both techniques tending to under-stage nodal status.[45, 46] Finally, Motohara et al.[45] reviewed the ability of MRI to detect extra-gastric metastases and concluded MRI had a greater sensitivity than CT in detecting liver, bone and peritoneal dissemination. The obvious advantage of MRI staging lies predominantly with its multi-planar capabilities, lack of ionizing radiation and use in patients with contrast hypersensitivity.[44] Other staging modalities include abdominal ultrasound, PET scans and staging laparoscopy.[36]

1.3 Surgical Therapy

1.3.1 Total, Subtotal and Proximal Gastrectomy

Choice of surgical procedure in resectable gastric cancer is dictated by size, location and ability to achieve surgical margins free of gross and microscopic disease. Several European studies have shown that to achieve adequate margins clear of disease,

there must be a 5 cm distance from the tumor to the closest resection line in intestinal – type and 10 cm margins in diffuse-type tumors.[39, 47-49]

In general, tumors confined to the proximal third of the stomach are treated with total gastrectomy to ensure adequate resection margins. It is controversial whether proximal gastrectomy is associated with poor functional outcome of the distal gastric remnant compared to a total gastrectomy with reconstruction. Although there are few studies to address this issue, Harrison et al.[50], in a retrospective review, demonstrated that patients with proximal gastric cancer who underwent total gastrectomy or proximal gastrectomy had similar overall survival times and recurrence rates. This study suggested both procedures could be accomplished safely. The authors suggest, although the two procedures are equivalent from a survival and recurrence perspective, further studies are necessary to assess nutrition and quality of life. Studies have demonstrated improved quality of life in the subtotal gastrectomy over the total gastrectomy group[51-53]; however, only one study [53] specifically demonstrated a reduced quality of life of proximal gastrectomy over total and subtotal gastric resections.

There remains controversy surrounding the choice of procedure for tumors of the middle and distal thirds of the stomach. In a large European survey involving 62 centers, Heberer et al.[54] demonstrated that 44% of surgeons prefer a total gastrectomy for diffuse-type gastric cancer of the antrum, based upon improved tumor clearance and local recurrence rates. In an analysis of 6400 patients in the US National Cancer Database, Hundahl et al.[13] showed that 12.3% of patients with cancer of the antrum or pylorus, regardless of tumor type, were treated with total gastrectomy. In a multi-center randomized trial of 618 patients, Bozzetti et al.[1] concluded that patients with cancer of the middle and distal third of the stomach, who underwent either subtotal or total gastrectomy, had the same 5-year survival. This study showed subtotal gastrectomy had shorter hospital stays, better nutritional status, fewer complications and better quality of life.[1] Furthermore, total gastrectomy had higher splenectomy rates with increased post-operative complications and susceptibility to infection, supporting the role of subtotal gastrectomy where possible.[1] The authors concluded that should a gastric cancer involve adjacent organs, these organs should be removed *en bloc* with the stomach, provided a combined procedure achieves clear resection margins.[47, 48]

1.3.2 Limited versus Extended Lymphadenectomy

The incidence of lymph node involvement ranges from 3-5% for tumors limited to the mucosa, 16-25% for those limited to the submucosa, and 80-90% in patients presenting with stage III or IV disease.[11, 55] Controversy exists regarding the appropriate extent of lymph node dissection (LND). Retrospective studies from Japan, involving more than 10,000 patients, suggest extended LND combined with gastrectomy prolongs survival compared with limited LND.[56-59] The extended LND produced overall 5-year survival of 50-62% versus 15-30% obtained for limited resections in the United States.[10, 58, 60] Japanese investigators assert that the extended LND (D2) removes tumor in the regional lymph nodes before it can metastasize. In addition, it is argued that extended LND improves staging accuracy.[55-59]

The discrepancy in overall survival rates between Japanese and western centers following extended LND led to two large multicenter randomized prospective trials. The Dutch Gastric Cancer Group[61] randomized 711 patients (380 to limited [D1] and 331 to extended [D2]) to undergo resection with curative intent. This trial showed that patients in the D2 group had a significantly higher rate of post-operative complications than did those in the D1 group (43% vs. 25% [$p<0.001$]), more post-operative deaths (10% vs. 4% [$p=0.004$]), and longer hospital stays (median, 16 vs. 14 days [$p<0.001$]).[61] Furthermore, the 5-year survival rates were similar in the two groups (45% in the D1 group and 47% in the D2 group).[61] In the Dutch trial, the authors noted stage migration occurred in 30% of the D2 group, and may have explained the east versus west difference in survival in patients matched for stage.[61] The authors concluded the results did not support the routine use of D2 LND. However, in a subgroup analysis, they showed a significant difference in patients with stages II and IIIA offered a D2 resection; an observation supported by Siewert et al.[62] Furthermore, Hundahl et al.[13] examining the mature results of the Dutch Trial, noted a risk of recurrence greater in the D1 than in the D2 group (41% vs. 29%; $p=0.02$), supporting the role of an extended lymph node resection.

Cuschieri et al.[63] conducted a randomized comparison of D1 (n=200) versus D2 (n=200) resections for potentially curable advanced gastric cancer in the Medical Research Council (MRC) trial. The results of the trial demonstrated a significant difference between the D2 group and the D1 group in post-operative mortality (13% vs.

6.5% [p=0.04]) and morbidity (46% vs. 28% [p<0.001]), with no difference in overall 5-year survival for D2 versus D1 (33% vs. 35%).[63] Similar to the Dutch trial, the MRC demonstrated no survival advantage with the classical Japanese extended resection; however, a subgroup analysis of the MRC trial demonstrated several interesting results. First, the greatest contributing factor to post-operative morbidity and mortality in the D2 group was the addition of a pancreatico-splenectomy (HR=1.53; CI 1.17-2.01).[63] Second, preservation of the pancreas and spleen with an accompanying D2 resection may carry a better survival than a D1 resection and can be carried out with low post-operative morbidity and mortality.[63] Interestingly, in both the Dutch and MRC trials, when a minimum of a D1 resection (removal of at least the N1 level nodes) was mandated for all patients, the overall 5-year survival of the D1 group jumped from a 20% survival to 34% (MRC) and 45% (Dutch), again suggesting a strong association between survival and an adequate LN dissection.[13] Cuschieri et al.[63] concluded that a "D2 resection without pancreatico-splenectomy may be better than a standard D1 resection, and cannot be dismissed by the results of this trial."

Several follow up studies based upon the Dutch and MRC results have examined the role of extended LND with pancreas and spleen preservation on post-operative morbidity, mortality and overall survival.[10, 55, 56, 62, 64-69] These studies demonstrated extended LND with preservation of the spleen and pancreas can be performed with post-operative morbidity and mortality equivalent to limited LND. Several well conducted prospective studies[10, 62, 64-66, 69] demonstrated extended LND is not associated with an increase in morbidity or mortality when conducted in experienced centers and markedly improves long-term survival in patients with stage II, IIIA[10, 62, 64-66, 69] and perhaps IIIB disease.[10] Based upon these studies, gastrectomy with extended lymph node dissection remains the procedure of choice in specialized centers.[56, 69-73]

1.3.3 New Issues with Lymphadenectomy for Gastric Cancer

Early editions of the TNM staging criteria were concerned with N status as defined by the location of lymph node (LN) metastasis relative to the primary tumor.[74] This created controversy with respect to appropriate lymph node resections, and prevented generalizability with Asian studies staged with the Japanese Classification for Gastric Carcinoma (JCGC).[75] The JCGC categorized the extent of LN metastasis on the

basis of anatomical LN station (Appendix 1). The presence of metastasis to each LN group reflects the N status and forms the basis of the D categories (Appendix 1).[74] With the recognition of the survival advantage of extended (D2) resections, the 5th edition of the AJCC TNM has been modified to include available clinical, radiological, endoscopic and surgical means to assess the extent of disease.[76] The 5th edition classifies LN metastasis based on the number of positive nodes, where at least 15 LN must be dissected and examined for staging to be accurate (Appendix 2).[75, 76] In a historical cohort, Karpeh et al.[75] demonstrated the number of positive nodes provided a better prognosis than anatomic location, as defined by an earlier TNM edition. Similarly, Kodera et al.[77] applied the 1997 TNM staging to 493 Japanese patients who had a D2 or D3 resection, and concluded the number of involved nodes was a strong prognostic indicator that should replace the N category in the JCGC. This finding has since been supported by several groups that similarly found increased LN number improves prognostication, minimizes the effects of stage migration, improves nodal staging across regions and countries, aids appropriate multimodality therapy selection and provides a better indication of disease burden.[75, 76, 78] In 1995, pathological N stage was defined by the number of metastatic LN, thereby achieving a single uniform staging system.[75]

Although not completely accepted, there is increasing consensus that retrieving at least 15 LN is necessary to accurately stage a tumor. However, there is considerable non-compliance by North American and European surgical centers. Mullaney et al.[76] showed only 31% (range 10-44%) of surgically resected cases could be accurately assessed for lymph node status. The paucity of LN for staging has implications for both prognosis and stage migration.[76] This observation was supported in a study that examined 1,038 patients in a single American institution and found that up to 27% of cases had fewer than 15 nodes examined.[75] Even more alarming was the report from the US National Data Base, which demonstrated that as few as 18% of US patients have $\geq$ 15 LN analyzed.[13] The authors suggest there is a high likelihood of residual, untreated regional lymph node disease in these patients. Non-compliance may be a failure in acceptance of extensive resections to improve prognosis, lack of familiarity with the extent of resection necessary to achieve the minimum LN count and inadequate pathological assessment.[13, 75, 76, 78]

1.4 Adjuvant or Neoadjuvant Therapy

Patients with localized node negative gastric cancer have 5-year survival rates that approach 75% when treated with surgery alone.[79] This is in contrast to patients with lymph node involvement, where survival rates range from 10-30%.[9] The outcome of gastric cancer is complicated by a high incidence of local recurrence and distant metastases following curative surgery, and has prompted interest in adjuvant therapies in the hope of improving treatment outcome.[58] Studies of adjuvant and neoadjuvant therapy in the treatment of gastric cancer have produced conflicting results. The inconsistency may be a reflection of the differences between populations studied (high vs. low risk groups)[80], pathologic classification[81], extent of surgical procedure (D2 vs. D1)[68] as well as differences in the content and timing of adjuvant therapy (immediate versus delayed). Several meta-analyses[82-88] have been published in attempt to address discrepancies reported in the literature, the findings of which are summarized in Appendix 3.

Three of seven meta-analyses suggest a small but significant advantage of adjuvant chemotherapy in the treatment of completely resected gastric cancer.[83, 84, 87] However, these authors suggest the results be interpreted with caution, as the results are of borderline significance[83], and may be influenced by a series of biases.[84] This conclusion reflected an earlier report that reviewed the results of 43 randomized trials between 1967 – 1993 concerning all adjuvant therapies for gastric cancer, including those published in the Japanese literature.[89] This review concluded that the results from North American and European randomized trials did not support the routine use of adjuvant chemotherapy for gastric cancer.[89]

Janunger et al.[85] in a systematic overview of 153 scientific papers (involving 12,367 patients) examined the effects of adjuvant chemotherapy in gastric cancer. In their meta-analysis, a significant overall survival benefit was demonstrated (Table 3). However, separate analysis of Western and Asian studies demonstrated a significant difference in outcome in Asian (OR 0.58, 95% CI 0.44-0.76) but not in Western (OR 0.96, 95% CI 0.83-1.12) reports; a difference attributed to timing of diagnosis, extent of surgery and stage migration.[85] In a more recent meta-analysis, Junuger et al.[88], applying modern drug combinations over the last 10-years failed to demonstrate any significant

survival benefit (Table 3). Overall, there is insufficient evidence at present to recommend post-operative chemotherapy as standard adjuvant treatment in western centers.[82-85, 88]

Preliminary studies of adjuvant chemoradiotherapy showed promising results in patients resected with curative intent.[90, 91] The role of adjuvant chemoradiotherapy was examined primarily in the Intergroup 0116 trial[92] that randomized 566 patients with stage IB-IVM0 completely resected gastric or gastro-esophageal adenocarcinoma to receive surgery alone or surgery plus chemoradiotherapy (5-Fluorouracil + leucovorin followed by 45 Gy of radiation). The surgery alone arm fared significantly worse when compared to the adjuvant chemoradiotherapy arm in terms of relapse-free survival (HR 1.52, 95%CI 1.23 – 1.86) and death (HR 1.35, 95%CI 1.09-1.66).[14] The addition of adjuvant chemoradiotherapy improved median survival significantly (p=0.005) from 27 months to 36 months.[14] Distant relapse was the most common site of recurrence in the adjuvant group (33% vs.18%), while local recurrence was more common in the surgery-only group (29% vs. 19%).[14] Significant toxicity (Grade 3 or higher) was observed in the chemoradiotherapy group, with 3 patients (1%) dying of treatment related toxicity. Furthermore, although the surgical protocol recommended an extensive lymph node resection, less than 10% of patients received a formal D2 dissection, while 54% underwent a D0 dissection.[14] The authors conclude the greatest benefit of chemoradiotherapy may be in high-risk patients treated with inadequate D2 resections. Despite the results of this study some institutions recommend adjuvant chemotherapy alone in patients unable to tolerate radiotherapy; however the optimal regimen in this setting has yet to be defined.[93]

Neoadjuvant therapy (chemotherapy, chemoradiotherapy, radiation or immunotherapy, either alone or in combination given pre-operatively) has been used with locally advanced tumors and those with a high risk of recurrence despite apparently curative surgery. Resectability rates of 40-100% and potentially curative resections in 37-80% of cases have been reported.[85] However, only two randomized trials have addressed neoadjuvant chemotherapy therapy, neither of which convincingly demonstrates clear benefit.[93-95] Studies regarding adjuvant intra-peritoneal chemotherapy are similarly inconclusive and are not administered routinely outside the clinical trial setting.[85]

1.4.1 Unresectable Locally Advanced or Metastatic Disease

Greater than 50% of patients present with unresectable locally advanced or metastatic gastric adenocarcinoma.[96] The majority of patients, including those with early stage disease, develop metastases at some point during the course of their illness. Symptom palliation in this group of patients is paramount, and can be thought of in terms of either local and/or systemic therapy. Treatment of local symptoms includes palliative surgery, radiation and/or endoscopic procedures. In patients with metastatic disease, systemic chemotherapy is the only treatment modality that has demonstrated a significant improvement in survival.[88] In selected patients with good performance status, compared to best supportive care alone, combination chemotherapy has been shown to improve median survival by 3-9 months, as well as demonstrating improvement or maintenance of quality of life.[97-100] Numerous traditional single agent chemotherapy regimes have been studied, with a variety of combinations evaluated in phase III trials demonstrating response rates of 25-40%.[101] Despite the number of regimens evaluated no single combination regime has emerged.[88] Standard protocols in North America include epirubicin, cisplatin and continuous infusion 5FU (ECF)[102], cisplatin and 5-day infusion 5FU (CF), and etoposide, leucovorin and bolus 5FU (ELF).[103] Third generation combination regimens have incorporated newer agents such as irinotecan, oxaliplatin and taxanes, all of which are currently under phase II-III evaluation. Despite the use of traditional combination chemotherapy, median survivals rarely surpass 10 months.

1.5 Prognostic Variables

1.5.1 Stage

The pathological stage has consistently been shown to be of prognostic significance for both 5-year survival and local recurrence rates.[62, 104-106] Siewert et al.[62] in a prospective multicenter observation study, demonstrated a lymph node ratio greater than 0.20 (between positive and removed nodes) was the single most important independent prognostic factor ($p<0.0001$)), followed by residual tumor status ($p<0.0001$) and T category ($p<0.0001$). In a multivariate subgroup analysis of completely resected tumors (R0), they confirmed nodal status was the most important predictor, followed by T category.[62]

1.5.2 Grade

Grade refers to the degree of differentiation of tumor cells and has been shown to correlate with the aggressiveness of the neoplasm.[6] Pathologic grade classifies tumors into one of three categories: well, moderately, and poorly differentiated/anaplastic.[6] Although grade is routinely reported in pathological reports, the prognostic impact in gastric cancer remains to be elucidated, as several retrospective studies have failed to identify grade as an independent prognostic factor.[106-108]

1.5.3 Size

Size of the primary tumor, measured in greatest dimension, has been identified in several retrospective studies to be of prognostic significance.[9, 105, 106] Studies suggest increasing tumor diameter is associated with lymph node metastasis and 5-year survival. This was confirmed in a prospective randomized trial that demonstrated tumor size to be an independent prognostic factor in a multivariate analysis ($p=0.0002$; CI [1.3-2.2]) in patients with tumor free margins.[62]

1.5.4 Tumor Location

The influence of tumor location has several important implications in the treatment and prognosis of gastric cancer. Although there are studies which have shown no association between location and prognosis[105, 107-109], several studies have shown that gastric carcinoma of the proximal third of the stomach represents a distinct clinical entity with prognostic implications.[2, 9, 11, 105, 106, 110, 111] A recent study suggested proximal tumors have a higher frequency of larger size, extensive wall penetration, venous invasion, nodal metastasis, and more advanced stage, with an overall worse survival relative to distal tumors.[111] Proximal tumors may require a different surgical approach based upon a potentially different biological behavior.

1.5.5 Lymphatic and Vascular Invasion

The presence of tumor emboli within peri-tumor vessels and lymphatics has recently generated interest as a potential independent prognostic indicator. Studies have demonstrated that lymphatic vessel involvement is a statistically significant predictor of survival, and the presence of tumor emboli significantly influences tumor recurrence and death following curative resection.[72, 105, 110] Yokota et al.[110] found lymphatic invasion retained its significance (RR=11.43; CI 2.63-49.55) even in competition with other

significant variables in multivariate analysis. These findings were recently supported in a report by Hyung et al.[112] who reported a poor prognosis associated with advanced T stage and the presence of vascular invasion. Kooby et al.[113] similarly demonstrated, in adequately staged node-negative patients, vascular invasion was an independent negative prognostic factor and may be a predictor of biological aggressiveness.

1.5.6 Age and Gender

Neither age nor gender have been shown definitively to be of prognostic significance for death from recurrent or metastatic cancer.[62, 109, 114] Two small retrospective studies in a subgroup analysis identified age as a significant prognostic variable [105, 108], while in another study the influence of age was not of independent prognostic value.[114] This study determined that survival was determined by stage and completeness of resection.

1.5.7 Miscellaneous Factors

Several other factors have been implicated with increased local recurrence and decreased survival in gastric cancer. Putative tumor markers (p53, E-cadherin, CD-34, c-ErbB2, CA 72-4, CEA) have recently gained popularity as potential prognostic indicators for predicting tumor behavior.[111, 115-117] These markers are likely to gain importance as the field of gene-expression analysis continues to expand.[117] Other factors include tumor perforation, emergency surgery, and blood transfusion.

1.6 Survival Analysis and its Application to Gastric Cancer

The utility of determining the prognosis of a disease is two fold. Prognostication provides information to patients and clinicians of the future course and natural history of the disease and allows for comparative analysis of a given outcome between two or more populations.[118, 119]

Prognostic studies often involve comparisons between two or more groups of patients which differ with respect to their disease status. Survival curves for each group may be constructed and the respective curves compared by the Log Rank test.[118] Alternatively, multivariate models may be used to incorporate both time and the effects of multiple factors on the time to a given outcome into the analysis.[118] This analysis may be used to identify a combination of factors that best predict the prognosis in a group of patients or the effect of individual factors independently.

The methods of survival analysis have been widely applied to the study of gastric cancer to determine the significance of prognostic factors in guiding clinical decision-making. Recently, survival studies have generated multivariate predictive models based upon clinicopathological factors and linked them to molecular pathways. This approach incorporates gene expression profiles, representing the biologic behavior of tumors, generated from microarray studies into predictive models and may be used to guide surgical and adjuvant therapy.

Chapter 2

Molecular Aspects of Gastric Adenocarcinoma

2.1 Future Directions in the Study of Gastric Cancer

Some epithelial cancers appear to follow the multi-step pathway of carcinogenesis. In these tumors, the correlation between genetic abnormalities and sequential phenotypic changes has allowed accurate clinical and pathological characterization.[36, 120-123] However, gastric cancer exhibits heterogeneity in histopathology and molecular changes that has impeded its complete molecular delineation.[121] Only a few genes (eg: c-met, c-erbB2, K-sam, E-cadherin) are implicated in gastric cancer.[124] Of these, only E-cadherin has been linked definitively, as a marker of Hereditary Diffuse Gastric Cancer.[25-27, 35, 125] As mentioned, most gastric cancers occur sporadically, with 8-10% having an inherited familial component. More commonly, gastric cancers occur without any consistent mutation abnormality. There is considerable variation in the pathogenesis ranging from a stepwise progression of changes (gastritis→ metaplasia→ invasive carcinoma), to tumors arising in the absence of a precursor lesion.[121] Novel technologies, such as microarray-based gene expression profiling, are providing information on the expression of many genes involved in human cancers.[126] This approach is promising to transform our understanding of the molecular interactions that ultimately describe a tumor phenotype and behavior.

2.2 Microarray-Based Gene Expression Profiling

DNA sequences do not tell us how gene-expression gives rise to phenotype or how gene-expression alters downstream molecular by-products.[127] Current limitations to understanding gastric carcinogenesis are techniques to link structural knowledge of genes to functional changes that occur between component parts; thereby providing insight into tumor behavior.[124, 127, 128] Characterization of genes that are differentially expressed in gastric cancer is essential for accurate diagnosis and tumor characterization and for informed surgical and adjuvant therapy decision-making, development of novel therapeutics and delineation of tumor behavior for more accurate prognostication.[124]

Microarrays have extended molecular research beyond the candidate gene approach and are beginning to establish a link between gene expression and functional interactions.[121, 124, 126-130] An advantage of microarray technology is that it is a

translational tool that incorporates functional interactions in an attempt to understand biology, not simply to identify the component parts of a pathway.[127] Gene expression studies allow characterization of genes that are differentially expressed or transcribed from the genomic DNA.[122, 124] The resulting collection of genes, referred to as the expression profile, is considered to be a major determinant of cellular phenotype and function.[127] Understanding the differences in gene expression between normal tissue and malignant tissue, as well as the gene expression response to environmental stimuli, is central to understanding regulatory mechanisms involved in cancer development and progression.[126, 127, 130]

2.3 The Evolution and Application of Prognostic Models in Gastric Cancer

Numerous studies have provided evidence for prognosis based upon either univariate or multivariate analyses of both clinical and pathological factors. The purpose of these investigations has been to develop a model capable of predicting the natural history of gastric cancer based upon a tumor's morphological and pathological make-up. The ability to identify a set of consistent predictors could allow surgeons and oncologists to treat gastric cancer and predict the outcome of therapy in a more consistent and informed manner.

Although providing insight into certain tumor characteristics, these studies derive from widely varying institutions, surgical practices, pathological nomenclature and staging systems, which has created confusion as to what method best predicts the biological behavior of gastric cancer. Nevertheless, studies having the greatest impact on surgical decision-making are those that address specific patient- and/or tumor-related issues such as the influence of age as a prognostic factor [131], subtotal versus total gastrectomy [1, 132], extended (D2) versus limited (D1) lymphadenectomy [56, 69-72, 133], the role of chemoradiation[14] and the importance of standardization and compliance with international guidelines in the treatment of gastric cancer.[76, 78] Awareness of the importance of standardization of surgical technique and staging nomenclature has facilitated the examination and comparison of prognostic factors across regions and countries. This has given researchers the opportunity to incorporate novel techniques into predictive gastric cancer models.

2.3.1 Development of Prognostic Models in Cancer Using Microarray Technology

Multivariate regression analyses have been applied extensively in the study of cancer. These studies have allowed the determination of a large number of important clinicopathological factors to guide clinicians with respect to management strategy. Despite this, traditional prognostic factors have limited predictive power and have changed current management strategies in only a few cancer types.[134, 135] However, microarray technology coupled with multivariate predictive models, has generated interest in the use of gene expression profiles as prognostic models.

Lymph node status, receptor status, proto-oncogenes and gene mutations have all been correlated to prognosis in breast cancer.[136] However, breast cancer is complex, and knowledge about individual prognostic factors provides limited information about the biology of breast cancer. Several recent studies linking novel gene expression data to multivariate prognostic models have been used to examine survival and to develop more precise markers of biological behavior to overcome the limitations of current predictive modeling techniques.[134, 136, 137] These studies have demonstrated how microarray analysis can accurately identify distinct subclasses of breast cancer [136, 137] and independently predict overall and relapse-free survival based upon "predictive gene-sets" that are superior to currently available clinical and histological prognostic models. [134, 137]

The application of microarray analysis to diseases such as non-small cell lung cancer, hepatocellular carcinoma, esophageal carcinoma and Barrett's esophagus have similarly shown the utility of microarray in documenting distinct prognostic groups, molecular staging systems, models capable of accurately predicting overall and disease-specific survival and recurrence rates beyond current techniques.[135, 138, 139] The application of gene expression profiles may therefore have the potential to refine diagnosis, prognosis and patient management.[138]

The majority of microarray studies examining gastric adenocarcinoma have been aimed at developing exploratory gene profiles of gastric tumor or gastric cancer cell lines to identify gastric cancer-related genes, delineate molecular phenotypes, demonstrate tumor subtypes and identify functional gene-clusters as potential markers of biological behavior.[124, 140-145] There are few studies that have applied combined microarray and predictive modeling methodology to gastric cancer. Recent studies have shown that

microarray, in combination with statistical modeling, accurately predicted tumor behavior with respect to tumor progression, metastatic potential, tumor recurrence and overall prognosis.[146, 147] Although in its infancy, gene expression analysis, combined with predictive models, holds promise in extending our understanding of gastric carcinoma. The relative paucity of data available relating gastric cancer gene profiles with prognosis and the success across various other cancers strongly reinforces the need for further exploration of this technique. With techniques capable of amplifying small quantities of tumor RNA, it is conceivable that small tissue samples obtained endoscopically or by needle biopsy may be used to generate pre-operative predictive gene-clusters.[148] In doing so, the identification of functional gene-clusters may allow improved selection of patients for neoadjuvant and adjuvant therapy, tailored surgical resections, identification of novel gene-clusters for targeted therapy design and improved prognostication to facilitate both clinician and patient decision-making.

2.4 Microarray Methodology in Cancer Research

The human genome project generated a massive number of small sequences of human genomic DNA termed Expressed Sequence Tags (EST).[121] The number of sequences currently deposited into the public database exceeds 3.5×10^6.[121, 128] The National Center for Biotechnology Information (NCBI) catalogs the sequences and reduces the dataset by collapsing overlapping sequences into a non-redundant set of expressed genes.[130] This has produced over 60,000 unique sequences, and has provided the starting material for global gene expression techniques.[121]

There are two general platforms for analyzing gene expression data using high-density microarrays: complementary arrays (cDNA) and oligonucleotide arrays.[127, 130] Both platforms employ a methodology in which a known sequence (probe) is deposited or synthesized *in situ* on a glass slide in a pre-defined grid pattern to which fluorescently labeled targets are hybridized[130] The amount of target hybridized to each probe is quantified using a confocal fluorescent microscope.[122, 128] Although both techniques permit simultaneous monitoring of the expression of thousands of genes in a single step, there are methodological differences.[128]

Construction of cDNA arrays involves the robotic deposition of nucleic acids (PCR product/cDNA probe) onto a 1.28 cm x 1.28 cm glass slide[122, 127] The cDNA probe

is derived from an EST database, each representing part of a human gene. Each gene or EST is typically a single double-stranded DNA probe up to 1,000 base pairs in length. The nucleic acid probe is generated from a polymerase chain reaction (PCR) using a cDNA library as a template.[127] Approximately 1-2 nanogram quantities of nucleic acid are then robotically deposited onto a glass slide coated with either poly-lysine or aminosilane that fix the probe to the slide[130] The cDNA probe is deposited at grid intervals of 100-300 *u*m.[127] Once on the slide, the double-stranded DNA is denatured into single strands. These strands are then available to serve as specific probes in experiments run as competitive hybridizations.[130]

Tissue gathered from a tumor is processed to extract messenger-RNA (mRNA). The target (mRNA) is labeled to allow quantification of gene expression.[127, 130] The mRNA is labeled by directly incorporating fluorescent nucleotide analogues into the cDNA during a reverse transcription (RT-PCR) reaction. Commonly used labels include the fluorophores Cy3 (or Cy5). [127] In cDNA platforms, a two-color hybridization strategy is employed. Copy-DNA from two conditions (experimental and reference RNA) are differentially labeled with two fluorescent dyes (Cy3 and Cy5), and the two samples are co-hybridized to an array. Determination of the expression ratios allows quantification of differential gene expression.[122, 127] Expression ratios are determined by scanning the array with a confocal microscope.

The creation of oligonucleotide arrays (oligoarrays) differ in a number of ways. Oligonucleotide probes may be deposited similar to cDNA arrays, or synthesized directly onto the platform surface in a grid pattern.[122] In the synthesized array, approximately 10^7 copies of selected oligonucleotide (usually 20-60 nucleotides in length) are synthesized onto a glass grid platform, with multiple probes per gene placed on the array.[127] Second, the oligoarrays do not require the maintenance of clone sets (cDNA libraries) since the probe is synthesized based on sequence data alone. The array is generated in situ using photolithography, allowing the fabrication of extremely high-density arrays.[130] Third, oligoarrays offer greater specificity than cDNA arrays since they are tailored to minimize cross-hybridization and include a uniform probe length. In the hybridization process, each target is hybridized to an array consisting of a series of oligonucleotides that have a perfect match-mismatch sequence allowing determination of the background noise.[130]

Compared to cDNA arrays, oligoarrays offer improved molecular recognition and hybridization and the ability to subtract background noise; this improves quantitative aspects and reduces false-positive results.[122, 130]

Once the oligoarray is constructed, the target (mRNA) is labeled either fluorescently or by generating an enzymatically amplified biotinylated-cDNA in a reverse transcription reaction.[127, 130] Competitive hybridization of the cDNA to the oligoarray is then carried out. Finally, the slide is scanned and quantitated in a manner similar to the cDNA arrays.[122, 127, 130]

2.4.1 Tissue Harvesting and RNA Extraction

Following surgical extirpation of a tumor, the tissue is processed through an aldehyde-based fixative, such as formalin. This processing preserves tissue and cellular architecture, allowing pathological diagnosis and staging of disease. Once fixation is complete, tissue blocks are taken from the area of interest and embedded in paraffin to maintain structural integrity and facilitate microscope slide preparation. Subsequent staining procedures allow characterization of the tissue based upon differential uptake/staining of cellular and stromal constituents. Formalin-fixed tissue blocks may be stored indefinitely.

The standard protocol for fixing and embedding tissue samples are not compatible with microarray experiments. Limiting factors include an inability to extract sufficient quantities of RNA from fixed tissue, formalin damages mRNA integrity and a delay between surgical devascularization and tumor processing leads to degradation of RNA by native tissue nucleases.[122, 149, 150] Recognition of the vulnerability of RNA has given rise to snap-freezing tissue samples for microarray studies.

Tissue harvesting begins when whole tumor specimens are transported within 30 minutes of surgical devascularization to a processing area. Since microarray experiments have a threshold for the quantity of starting molecular material, they typically require between 10-40 ug for oligoarrays and approximately 100 ug for cDNA arrays.[122, 128] This corresponds to approximately 100-mm^3 of viable tumor tissue, while avoiding necrotic or reactive fibrous tissue. The tissue block is stored at -80°C to prevent RNA degradation.[122] Once snap-frozen, a tumor sample may be stored indefinitely.

RNA is extracted from the stored tissue block using commercially available mRNA extraction kits. In general, the gastric cancer tissue and normal gastric epithelium are homogenized in a Trizol (Life Technologies Inc.) solution, and dissolved in RNase-free-water.[134] The total RNA is treated with RNase-free DNase to eliminate any contaminating DNA.[144] The RNA is precipitated out of solution in the presence of an alcohol and centrifuged. The RNA pellet is washed with an alcohol (70% ethanol) then dissolved in RNase-free water. Once isolated, the RNA is reverse-transcribed into a cDNA and labeled, or an amplified cRNA is generated by in vitro transcription using a T7 RNA polymerase.[134, 144, 145] In the latter case, a double-stranded cDNA is then synthesized from the amplified RNA.[141]

2.4.2 Microarray Data Analysis in Cancer Research

The basic premise of microarray experiments revolves around the hybridization of a fluorescently labeled target (mRNA) to an immobilized probe (single-stranded cDNA). If a gene is highly expressed, then a large number of targets corresponding to this gene will hybridize to its cDNA.[122, 151-153] Since the amount of probe on an array is assumed to be greatly in excess of the amount of target, the amount of binding of target to the probe is a function of the target copy number in the tumor specimen.[153] Therefore, the expression level of each gene in a tissue will produce a fluorescent signal proportional to the copy number of the gene.[152, 153] Determination of the expression ratio in a two-color system allows quantification of differential gene expression.[122, 127]

The expression ratios are computed by scanning the array with a confocal microscope at two different wavelengths to detect the relative transcript abundance.[121] Computation separates the images into spots. The assumption is that the brightness of each spot on an image corresponds in a linear fashion to the amount of label at the spot on the array.[152, 154] Computer software is then used to count the pixel brightness at each spot to determine raw signal intensity.[152] Points between the array spots are similarly counted to calculate background intensity. The difference between the raw and background intensities produces a corrected estimate of gene expression of a particular transcript.[152, 154]

Computational analysis has centered on two approaches: unsupervised and supervised techniques. An unsupervised technique, or clustering, involves the aggregation

of data without prior knowledge of its structure. This simplifies the data by organizing expression profiles based upon genes that are strongly co-regulated. In doing so, clues to unknown gene function may be inferred from clusters of genes similarly expressed across multiple samples.[151, 155] The unsupervised technique has been proposed as a means of defining new disease subclasses, reducing and visualizing data, describing the relationship between clusters, or predicting the categorization of a new sample.[130, 155] Unsupervised methods may employ a variety of algorithms and, although beyond the scope of this discussion, include hierarchical clustering, principle component analysis, multidimensional scaling, and self-organizing maps.[156]

In contrast, supervised techniques are designed specifically to classify data into known groups.[155] The objective is to find the best set of genes to be used in the prediction and classification of tumor samples.[130] With this method, prediction generally refers to the classification of tumor samples by characteristics such as disease subtype, tumor stage, or response to therapy. Supervised techniques may provide diagnostic information, by distinguishing between similar-appearing tumors, or may be capable of predicting clinical outcome by incorporating known clinical data.[134, 136, 137, 143, 146, 155, 157]

2.5 Complementary Studies for Microarray Validation

Several methods, such as northern blots, real-time polymerase chain reaction after reverse transcription (RT-PCR), cDNA sequencing, and *in situ* hybridization, have been used to measure mRNA abundance, gene expression, and changes in gene expression.[127, 128] Microarray technology is a new concept that has allowed researchers to explore the expression signature of thousands of genes simultaneously. The generation of large quantities of information however and the probability of error in processing, technique, and data analysis demand validity testing prior to widespread acceptance of its application.[128]

2.5.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The main limitation of most quantitative techniques (northern blots, in situ hybridization, and RNAse assays) is their low sensitivity.[158] In contrast, reverse transcription polymerase chain reaction (RT-PCR) is a method for amplifying defined sequences of RNA and permits the analysis of different samples from as little as one cell in the same experiment.[159] It is a sensitive quantitative method and can be used to

compare levels of mRNA in different sample populations, characterize patterns of mRNA, discriminate between closely related mRNAs and analyze RNA structure.[158] RT-PCR is frequently used to verify microarray data at the RNA level.[127, 128] In general, RT-PCR has been facilitated by automated systems and well described protocols that have allowed the verification of genes identified through expression studies with high sensitivity and specificity.[128, 158, 159]

A recent variation of RT-PCR called real-time or quantitative PCR (TaqMan PCR; Applied Biosystems, CA) has been applied as a means of validating microarray gene expression profiles.[128, 141, 147, 151, 160] This technique is a high throughput method that increases the quantitative ability of RT-PCR by providing accurate and reproducible information on RNA copy number.[128] In short, real-time PCR uses a fluorogenic probe that is annealed to one strand of the target cDNA sequence between forward and reverse PCR primers. One primer is labeled at the 5' end (reporter fluorochrome) and one at the 3' end (quencher fluorochrome). As Taq polymerase extends the forward primer, its intrinsic 5' to 3' nuclease displaces and degrades the dual-labeled probe, releasing the reporter fluorochrome. Release of the reporter label produces a fluorescent signal proportional to the amount of PCR product generated in each cycle.[128, 158, 159] Real-time PCR simplifies and accelerates the process of producing reproducible quantification of mRNAs.

2.5.2 Immunohistochemistry (IHC)

In general, mRNA levels are related to the activity of cellular genes, and for most genes, changes in mRNA abundance are related to changes in protein abundance.[127] Identifying genetic abnormalities up-stream of functional protein products is attractive in its ability to detect cell states and gene activity. However, it has been established that mRNA abundance does not necessarily correlate with protein levels or with post-translational modifications known to be important in the regulation of proteins.[127] A recent study[128] demonstrated the correlation between mRNA and protein abundance to be less than 0.5, emphasizing the need for confirmatory studies, preferably at the protein level.

Paraffin embedded tissue blocks are a standard method of storing pathologic specimens. This technique allows preservation of cellular and stromal constituents, as

well as tissue architecture. Archival blocks represent huge repositories of readily available information that may be used for protein-based methods of correlating prognostic information and for confirmatory microarray analyses.[140, 161-163]

The most frequently employed protein-based technique is IHC. IHC typically involves microtome cutting of paraffin sections (~ 5-8 um) for plating onto microscope slides.[163] The slide undergoes a xylene de-waxing and rehydration process, followed by the application of either a specific dye or enzyme conjugated antibody known to react with cellular or stromal components.[164] The target can then be localized or grossly quantified based upon staining pattern or percent cells stained in each section. Conjugated dyes can be viewed directly, while the presence of enzymes can be demonstrated by an appropriate histochemical method.[165]

Previously, the examination of candidate gene expression by IHC in archival specimens relied upon the laborious process of preparing individual slides for each clinical sample. Given the vast amount of gene expression data generated with microarrays, this methodology is both time and cost prohibitive.[165, 166] A recent development is the utilization of tissue microarrays.[163, 165] This technique involves the localization of a histological lesion of interest on a donor paraffin block after sectioning and Hematoxylin and Eosin (H&E) staining. Core needle biopsies ranging from 0.6 mm – 2.0 mm are taken from individual donor paraffin-embedded tissue blocks and arrayed into a new recipient paraffin block.[165, 166] The cores are taken with the assistance of an arraying instrument (Beecher Instruments, MD, USA) allowing accurate sampling of the tissue block and plotting into the recipient block through an X-Y precision guide.[163] Cores are placed into a grid pattern at intervals of 0.7 mm – 0.8 mm, allowing up to 1000 different specimens to be arrayed in a single 45 x 20 mm recipient block.[163, 165, 166] Once the tissue array is constructed, sections (5-8 um) are cut and analyzed in a fashion identical to conventional IHC studies. Tissue microarrays provide an efficient method for evaluating novel genes identified through DNA microarray using material from tumor archives that are 10-20 years old. Several studies have used tissue microarrays to provide instant long-term follow-up of large cohorts by linking well characterized clinical and prognostic information from archival specimens to prospective DNA microarrays.[161-166]

2.5.3 Immunoreactivity Protein Targets

Multivariate modeling techniques have evolved to allow the assessment of multiple factors simultaneously.[118] This allows one to define the independent effect of one variable by adjusting for the effects of several other extraneous variables.[118] This has traditionally involved the assessment of clinical and pathologic factors related to clinical outcome. These methods may be extended to incorporate protein-expression profiles obtained through immunohistochemical studies. Several promising protein targets related to the biologic behavior of gastric cancer have been identified. Five protein targets previously found to predict local tumor infiltration (T status) and lymphovascular invasion (LVI) in gastric cancer were selected for inclusion into the present study.

i) Cyclooxygenase (COX): COX are key enzymes in the biosynthesis of prostaglandins (PGs) which are potent biologic mediators with both physiologic and pathologic effects.[167] Two isoforms of COX have been identified. COX-1 is constitutively expressed in most normal tissue and is responsible for normal renal and platelet function and the maintenance of the gastrointestinal mucosa. [168] COX-2 however is normally undetectable in most normal tissue and is induced by various stimuli such as cytokines, oncogenes and tumor promoters.[167, 168] Induction of COX-2 leads to the production of PGs with growth-stimulating properties. COX-2 expression appears to favor malignant growth by inhibiting apoptosis, promoting angiogenesis and inhibiting immune surveillance. [168] Several studies have demonstrated elevated expression of COX-2 in human tumors, including colon, breast, lung, esophagus and more recently stomach.[167-170] Murata et al.[170] demonstrated that COX-2 over-expression was detected in 70% of gastric cancers and was associated with enhanced lymphatic permeation, metastasis and poor overall prognosis. This data was supported by Han et al.[167] who demonstrated that constitutive expression of COX-2 increased the metastatic potential through the activation of the matrix metalloproteinase-2 (MMP-2) pathway. Similarly, COX-2 over-expression has been linked to local tumor invasion (T status) in gastric cancer, where increasing COX-2 expression was correlated with increasing depth of tumor penetration.[169]

ii) Matrix Metalloproteinase (MMP): Gastric cancer typically demonstrates extensive local tumor invasion, with subsequent spread to regional lymph nodes. This process is normally protected by a mechanical barrier in the form of the basement

membrane (BM). The BM is composed of various structural glycoproteins and fibrous proteins capable of regulating permeability. The fibrous protein is composed primarily of type IV and V collagen.[171] Invasion of the BM proceeds through a series of steps coordinated by the MMPs, able to degrade type IV and V collagen, allowing access to regional lymphatic and vascular channels.[172, 173 174]

Several studies have shown an association between protein-expression levels of various MMPs and local tumor invasion (T status)[172-175], lymphovascular invasion[171, 175] and overall survival[175, 176] In the present study, the protein-expression profiles of MMP-2 and 9 were examined for their ability to predict T status, LVI and disease-specific survival.

iii) Vascular Endothelial Growth Factor C (VEGF-C): Lymph node metastasis is the most important predictor of poor outcome in gastric cancer.[106] Lymph node metastases are strongly related to lymphatic and vascular invasion in the primary tumor.[177, 178] The metastatic process may be enhanced by the formation of newly formed leaky blood and lymphatic vessels through a process called neovascularization.[177-179]

VEGF-C is a glycoprotein with mitogenic properties on lymphatic endothelial cells, promoting the formation of newly formed lymphatic vessels.[180] Lymphangiogenesis contributes to the formation of poorly formed lymphatics within the primary tumor, leading to enhanced rates of lymph node metastasis and tumor angiogenesis.[180] Several studies have demonstrated a strong relationship between VEGF-C expression with LVI, lymph node metastasis and overall survival. [177-179, 181-183]

2.6 Challenges in the Application of Microarray Technology

2.6.1 Tissue Ischemia

The ability of DNA array technology to provide insight into gene profiles is dependant upon isolation of tissue and tumor mRNA. Several groups have raised concerns over the procurement of tumor RNA following surgical devascularization; whereby the tissue is exposed for variable lengths of time to the effects of warm ischemia.[149, 150] Degradation of RNA by native nucleases may have a profound effect on the expression profile and the quality of data derived from microarray analysis. Dash et al.[150], examining prostate cancer, identified the need to exclude spuriously deregulated genes because of artifacts introduced as a consequence of prolonged warm ischemia in

the procurement process. In a study assessing the time dependant effect of warm ischemia, Huang et al.[149] demonstrated that although temporal changes in RNA expression levels occur following tissue excision, snap-freezing of tissue within 20 minutes of vascular interruption provides relatively stable gene expression profiles. This observation was confirmed in a study which demonstrated little overall gene expression variability with ischemia within 1-hour of processing.[150] Despite these observations, until a method of in vivo sampling with processing that eliminates the effects of tissue ischemia is developed; all efforts should be aimed at minimizing tissue handling and at reducing warm ischemia time.

2.6.2 Tissue Heterogeneity

An ongoing point of contention with microarray analysis concerns the use of whole tumor sample versus laser micro-dissection of individual epithelial cells for harvesting of RNA. Proponents of whole tumor sampling argue tumors are by nature mixtures of different cell types, including malignant epithelial cells, stromal elements, blood vessels and inflammatory cells, all of which interact to produce an environment conducive to tumor existence and progression.[121, 122] Furthermore, interaction between malignant and non-malignant cells may play an important role in tumor expression signatures. Boussioutas et al.[121] have supported this notion, suggesting the use of clonal cancer cell lines are flawed since they have been removed from their in vivo environment, and are lacking the essential ingredients for tumor phenotype.

In contrast, others have focused on the technique of laser capture microdissection (LCM) to isolate malignant epithelium.[122] This process allows for the isolation of individual cells from a tumor section; thereby avoiding potentially confounding signals from adjacent tissue constituents. Despite the precision of this technique, RNA quality and quantity may not be amenable to microarray analysis.[184] Finally, the ability to differentiate tumors with similar clinical and phenotypic characteristics may depend upon a consideration of the proportion of different tumor elements.

Presently, there is no consensus as to which method is superior. Advocates of both groups are represented in the literature. As more information is translated from each technique, the utility of each method is likely to become clearer.

2.6.3 Issues with RNA Quantity

Microarrays depend upon the successful isolation and purification of high quality RNA from tumor samples. When starting with relatively large tumor samples, this is not usually a problem. However, as the clinical application of microarrays is expanded to include tissue biopsies obtained through endoscopy or needle biopsy, the availability of tissue may be a limiting factor. Furthermore, proponents of LCM may have difficulty in isolating sufficient numbers of malignant cells in tumors with predominant stromal reaction or for tumors that are relatively hypocellular, as may be encountered with schirrous-type gastric cancer.[127] A solution to this problem may involve PCR-based amplification of target RNA.[127, 160]

Lockhart et al.[127] found that although the PCR-based amplification was efficient and reproducible, the relative abundance of cDNA product is not well correlated with the original mRNA levels. A variation of this approach uses multiple rounds of linear amplification based upon cDNA synthesis and a template-directed *in vitro* transcription reaction.[127] This technique (T7-based RNA amplification) has been successfully applied to laser-captured cells from brain tissue for hybridization to spotted cDNA arrays.[185] The method involves RNA extraction and independent linear amplification an estimated 10^4 - 10^6-fold using a T7 RNA polymerase (Epicenter Technologies, Madison, Wisconsin).[127] Following amplification, the RNA may be transcribed, labeled, and hybridized to a microarray platform. Lockhart et al.[127] has demonstrated that sufficient quantities of labeled material may be generated from as little as 1 – 50 *n*g of starting total RNA. Similarly, Mori et al.[160], examining the progression of gastric cancer, was able to generate sufficient quantities of RNA for gene expression analysis with only 9 *u*l of starting total RNA. This study amplified extracted RNA 10^4-fold, demonstrating the utility of this method in overcoming issues of RNA quantity.

2.6.4 Reference Standards in Microarray Experiments

One of the difficulties with microarray experiments is the comparability of profiles between different experiments and between different laboratories. Holloway et al.[186] suggested that the adoption of a universal reference standard could overcome the difficulties associated with variation among studies and research groups. Although there is no consensus as to what constitutes a universal standard, it has been suggested that

pooled RNA derived from tumor cell lines or single cell lines may be an acceptable standard.[186] Difficulty with this approach however, is the possibility of batch-to-batch variation that may on its own represent an additional source of bias that may confound inter-experimental comparisons.

Alternatively, a reference standard may be generated by pooling either normal cells or tumor samples. This ensures that every sample present in the test sample will be represented in the reference sample and the relative amounts of each RNA species will be similar; thereby overcoming the inherent biological variability of the disease state.[130, 186-188] This technique has the advantage of avoiding discrepancies in RNA concentrations between samples.[188] Some authors have argued that reference samples are not necessary, and the practice of making comparisons to a reference standard introduces error, in a fashion similar to the use of multiple statistical tests.[188, 189]

2.6.5 Miscellaneous Problems with Microarray Preparation

Microarray is a complex methodology that is subject to biologic, technical, and analytical error. Several reports suggest that a wide natural variation exists in different disease states.[130, 188] This represents a non-modifiable factor that researchers must consider in interpreting array results. However, there are several technical steps in the preparation of microarrays that may introduce significant error and threaten the generalizability of the data. Although a complete discussion of each is beyond the scope of this study, recognition of the challenge each presents to microarray experiments warrants their introduction. One may classify error in microarray with respect to the tissue, the equipment, and the analysis.

Issues of tissue collection with respect to warm ischemia time, tumor sampling, tumor heterogeneity and difficulties with RNA quantity have been discussed. Difficulties with array equipment and construction may begin with an error in the source and identity of the clones used in the array. In an effort to identify clone identity, Halgren et al.[190] demonstrated only 62% of cDNA inserts had sequence identity with the published data. Based upon this finding, Pollock [187] stresses the need for independent confirmatory studies (RT-PCR, Northern Blot). Variations in dye intensity, efficiency of dye incorporation, and direct dye labeling have been identified as sources of error. These problems may be overcome with more efficient reverse transcriptases or by employing

indirect amino allyl labeling methods that circumvent the need to incorporate bulky fluorescent dyes during transcription.[186] Variability in the efficiency of hybridization and the concentration of DNA deposited at each spot are potential issues associated with the experimental conditions and reagents utilized. This may be overcome by employing commercially available hybridization kits and optimizing and standardizing experimental conditions to enhance reproducibility. Finally, Holloway et al.[186], have suggested additional sources of error may exist in signal intensity based upon slide selection and the choice between cDNA and oligonucleotide arrays, and therefore urges caution when comparing different samples among and between test sets.

Errors with informatics range from data acquisition, storage, software employed and choice of analytic method.[128, 191] Reliability and interpretability of array data depends upon appropriate selection of data for analysis, validation of results and careful consideration of the research objectives.[191]

Chapter Three

Population-Based Gastric Cancer Model

3.1 Introduction

Despite a declining incidence, gastric cancer is the second most common cancer worldwide and the third commonest cause of cancer deaths in Canada.[5, 19] Studies examining the biologic behavior of gastric cancer have relied upon clinicopathologic characteristics as a means of establishing prognosis.[3, 63, 106] With a trend toward a standardized approach to gastric cancer, complete delineation of the predictors of biologic behavior in guiding surgical decision-making becomes increasingly important.[76, 78] Specialized oncology centers have played a key role in establishing prognostic predictors in western populations.[61, 62, 68, 70, 75] Expertise and adherence to standard technique creates a reference for population-based studies, thereby improving quality and compliance with standardized surgical and pathological techniques.[76, 78]

Since most gastric cancer surgery is conducted in non-specialized centers in Canada, it is important to validate the results from specialized centers in population-based studies. Population-based studies overcome the issues of selection bias encountered by specialized hospital units.[9, 192-195] Population-based studies, by including all diagnosed cases, address surgical/pathological and patient heterogeneity, thereby allowing comparisons of survival estimates between geographically defined populations.[9, 193, 195] Identifying predictors of outcome from population studies may be used to guide management strategies and provide a platform from which future hypotheses can be generated and tested.[192]

We conducted a retrospective study to identify independent predictors of survival in a population-based cohort of Northern Alberta residents diagnosed with gastric adenocarcinoma. We present the results of a multivariate analysis, as well as a subgroup analysis of the relationship between tumor thickness (T status) and lymphovascular invasion (LVI).

3.2 Objectives and Hypotheses

The objective of this portion of the study was to determine clinicopathologic factors predictive of disease-specific survival for persons with gastric cancer, and to

compare the findings with those from similar large-scale international population-based studies. The specific objectives were both descriptively and analytically based.

3.2.1 Descriptive Objectives

i) To describe the distribution of potential prognostic variables in a retrospective cohort of patients presenting with primary gastric cancer (n = 577).

3.2.2 Analytic and Methodologic Objectives

i) To establish a comprehensive population-based gastric cancer database to determine prognostic factors for overall survival through the application of multivariate analyses.

ii) To examine the relationship between demographic, intraoperative, tumor/pathologic-specific factors and outcome in gastric cancer.

iii) To quantify the risk of different prognostic factors on outcome in gastric cancer.

3.2.3 *A Priori* Hypotheses

The following hypotheses were established *a priori*:

i) Tumor thickness, lymph node status, metastatic disease, residual tumor status, esophageal/duodenal margin status and tumor histology are significant prognostic factors associated with poor disease-specific survival in patients with gastric cancer.

ii) LVI is associated with aggressive behavior and is associated with worse disease-specific survival.

iii) LVI is a more important predictor of survival than tumor thickness.

iv) Important prognostic factors identified from a large (n = 577) population-based cohort of patients with gastric cancer are consistent and generalizable with similar international studies.

3.2.4 Research Hypotheses

i) Construction of a population-based prognostic model will facilitate comparison between similar prognostic studies examining gastric cancer.

ii) The generalizability of the prognostic factors identified will facilitate the application of predictive gene expression profiles in gastric cancer.

3.3 Materials and Methods

3.3.1 Study Design

This portion of the study is based upon a retrospective cohort design. To allow for a minimum follow-up of 5 years, patients having a diagnosis of primary gastric cancer from January 1, 1991 to December 31, 1997 inclusive were considered eligible for the study (Retrospective, Group I). Follow-up continued until August 30, 2003. Inclusion into the cohort included any patient registered with gastric cancer through the Alberta Cancer Board (ACB) in Northern Alberta (n = 1.57 million residents). Group I patients who received surgical treatment in any of the four major hospitals during the inception period have representative archival paraffin-embedded tumor samples stored within either the University of Alberta Hospital (UAH) or Dynacare-Kaspar Medical Laboratories (DKML, Edmonton, AB) archives. Selected paraffin blocks representative of the primary gastric tumor were obtained following ethical approval to allow immunohistochemical analysis and correlation with subsequent molecular studies.

3.3.2 Selection of Retrospective Study Cohort

All patients having a diagnosis of primary gastric adenocarcinoma registered with the Northern Alberta Cancer Registry from January 1, 1991 to December 31, 1997 were reviewed for potential inclusion (Group I). All diagnoses related to gastric malignancies were identified using ICD-0 codes based upon site of malignancy (C-16), followed by histological classification (WHO criteria) for gastric adenocarcinoma (8140, 8144, 8145, 8260, 8211, 8480, 8490, 8020).[19] Histological codes were obtained from final pathological reports. Patients identified included all patients admitted to one of the four Edmonton hospitals (University Hospital, Royal Alexander, Grey Nuns' and Misericordia) in addition to any patients in the Capital Health referral area. Provincial legislation mandates that all patients in Northern Alberta with a diagnosis of gastric cancer are registered with the ACB.

3.3.3 Inclusion and Exclusion Criteria

Medical records pertaining to Group I patients identified through ICD-0 codes were assembled from the ACB medical records department and reviewed for inclusion into the study. The following inclusion and exclusion criteria were applied to the retrospective cohort:

Inclusion Criteria:

i) A diagnosis of primary adenocarcinoma of the stomach as reported in the final pathology report.

ii) Primary tumor arising within any portion of the stomach (pylorus, antrum, body, fundus or cardia).

iii) All patients undergoing surgical therapy (curative or other) during the specified time interval.

Exclusion Criteria:

i) Operation for recurrent disease.

ii) Surgical therapy for disease other than adenocarcinoma of the stomach.

iii) Patients residing outside of the Northern Alberta Cancer Registry referral area.

Group I includes all patients with a diagnosis of adenocarcinoma of the stomach, includes patients treated for cure, palliative surgical procedures, surgical diagnostic procedures, endoscopically diagnosed patients and patients referred to the Cross Cancer Institute for neoadjuvant and adjuvant therapy (curative or palliative). Since Edmonton is a major referral centre for all of Northern Alberta and Northern British Columbia, Group I represents a population-based cohort.

3.3.4 Cross Cancer Institute (CCI) Records

All patients with a histologically confirmed diagnosis of cancer are entered into the Alberta Cancer Registry, which is linked to Vital Statistics at Alberta Health. By law, a death and cause of death in the province of Alberta are reported to the CCI and Alberta Health. All patients in the Alberta Cancer Registry have a patient file at the CCI containing information regarding name, age, gender, date of diagnosis, diagnostic code (ICD), treating physician, therapy offered at the CCI, and survival status. The Registry and patient file are updated for death on a monthly basis. Additional information regarding patient status (disease-free, alive with metastasis) is updated yearly.

CCI charts of all patients included in the study (n = 577) were identified by ACB number, name and birth date. These charts were reviewed by a single investigator (BD) for additional clinicopathological data. Review and recording of these data were completed in a blinded fashion by using a separate data collection form to limit potential outcome bias. Clinicopathologic data was ascertained for all 577 patients. Missing data

for any given variable was included in the gastric cancer database and designated "88" to ensure complete data collection. Similarly, patients having undergone surgical therapy but found to be unresectable were included by a designation of "99" in the gastric cancer database. The CCI data constituted the raw database and included data from 577 patients.

3.3.5 Clinicopathologic Sources of Data

Medical charts of all patients meeting the inclusion/exclusion criteria were reviewed and data regarding demographic, surgical operative notes, radiological reports, pathological synoptic reports and follow-up data. This data supplemented the CCI database. Data collection for Group I was considered complete once ascertainment was made of all clinicopathological and follow-up variables as described below. Data collected was done prior to ascertainment of patient status.

3.3.6 Clinicopathologic Data Collection and Instruments

A coded data collection form was used for all patients (Appendix 4). A summary of all variables and their coding is shown in Appendix 5. A Medline search between 1970 and 2002 of the English-speaking medical literature was undertaken using the keywords "gastric", "adenocarcinoma", "prognosis", "outcome" and "predictive models" to identify relevant articles. Sixteen clinicopathologic factors were identified as potentially important predictive variables in primary gastric adenocarcinoma. These variables were incorporated into the data collection form prior to accessing the CCI and hospital medical records. A list of the putative prognostic variables and definitions are included below.

3.3.7 Potential Prognostic Variables

i) Age – entered as a continuous variable, subsequently coded as categorical variable after demonstrating a lack of linearity.

ii) Gender

iii) Tumor thickness (T status) – Appendix 2

iv) Regional Nodes (N status) – Appendix 2

v) Metastatic disease (M status) – Appendix 2

vi) Stage (TNM) – Appendix 2

vii) Tumor Morphology – histological classification of primary tumor as defined by the World Health Organization (Appendix 2, section 2.1)

viii) Tumor Diameter (cm) – defined as longest transverse diameter on the gross specimen, measured to the nearest centimeter. On statistical analysis, tumor diameter was found not to be linear and was subsequently codes as a categorical variable.

ix) Lymphovascular invasion – the presence of tumor emboli/cells within peri-tumor blood vessels, within lymphatic channels or the presence of tumor cells in a peri-neural distribution. Lymphovascular invasion does not refer to lymph node involvement as per the TNM staging.

x) Tumor Grade – differentiation of the primary tumor or the degree with which the tumor differs from normal gastric epithelium. Grading is scored into one of three categories (well, moderate, or poorly differentiated).

xi) Proximal resection margin (esophagus) – pathological and gross examination of the resection margin. This is measured in centimeters from the tumor to the proximal esophageal margin. Margins are reported as positive (presence of viable tumor cells at the resection margin) or negative (no evidence of viable tumor cells).

xii) Distal resection margin (duodenum) – reported in nearest centimeters and pathologic scoring as above from the distal duodenal margin.

xiii) Type of surgical resection performed – refers to the surgical resection carried out (subtotal/total gastrectomy, surgical bypass, laparotomy only).

xiv) Chemotherapy – includes neoadjuvant and adjuvant therapy and single or combined modality

xv) Radiotherapy – includes neoadjuvant and adjuvant therapy and single or combined modality.

xvi) Ratio of positive lymph nodes to resected lymph nodes – represents a ratio of the number of lymph nodes harboring viable tumor cells to the total number of lymph nodes resected and examined.

xvii) Outcome event – refers to death from gastric cancer. All non-gastric cancer deaths are treated as censored cases. The outcome of interest is disease-specific survival (from inception to death from gastric cancer). Overall survival refers to the time from inception to death from any cause.

3.3.8 Clinicopathologic Data Management

Data was entered into a statistical spreadsheet (SPSS, Chicago, IL) on a Hewlett-Packard 1180 personal computer. The data was numerically coded and omitted personal and hospital identification numbers, but included a unique identifier for cross-reference to the original data collection form.

The structure and personnel of the Alberta Cancer Board Registry ensures strict quality control over data entry. Despite the quality of the registry, four methods of data cleaning and validation were carried out on the completed database. An initial complete independent audit of all patient charts was undertaken, and cross referenced to the existing Registry data. Second, exploratory descriptive statistics were used to identify missing data, incorrectly entered data and obvious outliers. This method identified inaccuracies and missing data primarily in the entry of T, N, M and stage. Suspect charts were again reviewed and necessary corrections to the database undertaken. Third, cross-tabulation among multiple related categorical variables was applied and resultant discrepancies identified. Finally, random case selection and data verification was performed. All data entry and analysis were performed on a single personal computer protected by a security password. Data will be retained for possible long-term follow-up studies after completion of this study. This file will remain anonymous with respect to patient name and hospital identification.

3.4 Statistical Methods

Analyses were undertaken with SPSS statistical software, version 11.0 for Windows (Chicago, IL). A p-value of < 0.05 was considered statistically significant.

3.4.1 Univariate Analyses

All univariate analyses employed the following tests:

i) ***Chi-square test*** for categorical data was used to test differences between proportions and to assess the linear association between independent variables.

ii) ***Kaplan-Meier*** method was used to obtain survival curves. The log-rank test was used for univariate analyses of these outcomes. This test assumes that the two groups under investigation (dead versus alive) are independent random samples, and censoring patterns for the observations are the same for the two groups.

3.4.2 Multivariate Analyses

i) ***Cox proportional hazard*** regression was used to determine the adjusted associations between independent variables identified through univariate analysis with time-related outcomes (survival).[196] The general model is as follows;

$$h_i(t)/h_0(t) = \exp\{B_1X_1 + B_2X_2 + B_3X_3 + \ldots + B_iX_i\}$$

$h_i(t)$ is the hazard of the dichotomous outcome at time t, $h_0(t)$ the baseline hazard dependent only on time, B_i the unknown coefficient for the i^{th} independent variable and X_i the independent predictor variable.

The multivariate models were examined by means of a purposeful regression with independent variables entered or removed based on the significance of the likelihood ratio test.[196] The reduced model was fitted against the full model to assess the significance of the removed variables with the likelihood ratio (LR) test. Potentially important variables removed were assessed for confounding through observed changes in the regression coefficients (β) of the variables retained in the final model. A variable was considered to be an important confounder and retained in the final model if it changed at least one (final) model β coefficient by > 15%. Continuous variables were assessed for linearity, and replaced with categorical variables where appropriate. No interaction terms were statistically significant, and they were not included in the final model. Pairs of variables demonstrating high collinearity were assessed and the variable with lower clinical importance was dropped from the final model.

The model is based on the assumption of proportional hazards, where the hazard ratio is a constant over time or the effect of the covariate does not change over time. This assumption was tested for all proposed variables entered into the final model. This was tested by plotting a log minus log survival (LML) plot of Kaplan-Meier survival curves. If the resultant plots appeared parallel, the proportional hazards assumption was considered valid.

3.5 Ethical Considerations

Ethics approval for this study was obtained from both the University of Alberta Health Ethics Research Board (Appendix 8) and the Cross Cancer Institute Research Ethics Committee (Appendix 9).

3.6 Results

3.6.1 Clinical and Operative Characteristics

The clinical and operative characteristics of the study cohort (n = 577) are shown in Table 3.1. The study cohort had a mean age of 72.6 years (range 27 – 106 years) at the time of surgery, and consisted of 344 (60%) males and 233 (40%) females. There was no association between in-hospital mortality and advanced age at the time of surgery ($p = 0.14$), nor was there a difference in age ($p = 0.27$) or survival ($p = 0.47$) between males and females in the study cohort.

Surgical therapies with curative intent included patients having a total gastrectomy (16.5%) or subtotal gastrectomy (38%). Palliative surgery was performed in 80 (14%) cases and resulted in 29 (5%) surgical bypasses and 51 (9%) laparotomy-only. One hundred eighty-three (31.7%) patients with a histological diagnosis of gastric cancer were unresectable. Nineteen (10.4%) of 183 patients were unresectable based upon pre-operative investigations and were not offered surgical intervention, while 164 (28%) of the entire cohort (n = 577) were found at surgery to be unresectable. There was a significant association between perioperative mortality and patients found at surgery to have unresectable disease ($p < 0.001$).

Surgical resection margins were assessed pathologically and divided into proximal (esophageal) and distal (duodenal) margins. Margins were considered negative in the absence of tumor cells within 1 cm of the resection margin and positive where tumor cells were identified at the resection margin (Table 3.1).

Adjuvant therapy was offered to 94 (16.3%) of 577 patients. Forty-two (7.3%) received chemotherapy and 52 (9.0%) received radiotherapy. There were 57 (9.9%) hospital mortalities recorded following surgical intervention. There were no intra-operative mortalities.

3.6.2 Tumor Characteristics

Tumor characteristics are presented in Table 3.2. Thirty-seven (6%) had T_1 tumors, 57 (10%) T_2 tumors, 202 (35%) T_3 tumors and 101 (18%) T_4 tumors, while 164 (28%) were unresectable at operation and therefore had incomplete T staging. Overall, 94 (18%) were N_0, 183 (35%) N_1, 89 (17%) N_2 and 16 (3%) N_3 tumors. Distant metastasis was absent in 125 (22%), present in 216 (37%) cases while in 236 (41%) cases distant

metastasis was not assessed. Mean tumor size was 5.9 cm (range 0.5 – 19.0 cm). Lymphovascular invasion was reported in 250 (43%) and absent in 92 (16%) patients. LVI was not recorded in 235 (41%) cases. Of the cases where LVI was not recorded, only 88 (15%) patients had surgical specimens for pathologic review, while 147 (25.5%) were found to be unresectable and therefore did not have adequate tissue to allow LVI to be assessed. At least 15 lymph nodes were resected in 199 (34%), while the remaining 378 (66%) were classified as unresectable or inadequately staged (Table 3.2). Tumor histology was defined according to the World Health Organization classification of tumors.[19]. The majority of tumors (54%) were classified generally as adenocarcinoma, with no morphological sub-classification.

3.6.3 Population-based Outcomes

Median follow-up for the entire cohort was 58 months (range 1 – 108 months). At the time of analysis, 81 (14%) patients were alive, 492 (85%) had died and 4 (1%) were lost to follow-up. Crude survival rates measure the number of events divided by the total study population (n = 577), while disease-specific survival rates measure the number of events directly attributable to gastric cancer. Five-year survival rates are provided to facilitate comparison to the literature. The 5-year crude survival rate was 12% (95% CI: 8.9 – 14.9, Fig. 3.1). The overall 5-year disease-specific survival was 28% (95% CI: 21.9 – 33.2). Five-year disease-specific survival according to AJCC/UICC TNM classification and median survival is presented in Table 3.3.

Population-based prognostic factors were assessed by univariate Cox's regression (Table 3.4). Clinicopathologic factors found to be significant by univariate analysis were entered as independent categorical variables into a Cox's proportional hazard model, and examined in a step-wise purposeful selection method. Criterion for entry into the Cox model was a $p < 0.10$ of the likelihood ratio statistic to reduce the possibility of a variable of borderline significance being excluded from the final model. Variables deleted did not contribute significantly to the final model ($LR = 9.12$, $p = 0.33$). All deleted variables were assessed for two-way interaction and confounding. No factors examined for interaction had a p-value < 0.01. Tumor size was the only non-significant predictor of survival included in the final model, as it was found to be a clinically significant confounder. Duodenal margin status was judged to be statistically significant; however, it

demonstrated collinearity with esophageal margin status. Given the clinical importance of esophageal margin status, it was retained while duodenal status was dropped from the final model. The proportional hazard assumption was met by each variable in the final Cox model as assessed through LML plots. Two separate Cox models were examined: the first contained T status, N status and M status as separate variables (Model A), while in the second model (Model B) tumor stage was used instead of T, N and M status. Apart from the mentioned differences (T, N, M vs. Stage), Cox's regression produced identical covariates predictive of long-term survival in the main effect models (esophageal margin, tumor histology, R-status). Model "A" was selected as the study model based upon the amount of clinical and predictive information provided by examining the components parts of stage individually (Appendix 2).

The final Cox's proportional hazard model is presented in Table 3.5. Nodal status was the most significant independent prognostic factor ($p < 0.001$, Fig. 3.2), followed by T status ($p < 0.001$, Fig. 3.3), histological classification ($p = 0.002$), esophageal margin status ($p = 0.01$, Fig. 3.4), residual tumor category ($p = 0.01$) and M status ($p = 0.03$). Tumor size was not significant ($p = 0.13$), but found to be a confounding variable and therefore included in the final model as an important predictor of survival.

No significant 5-year survival difference was observed between T1 and T2 tumors ($64 \pm 18\%$ vs. $53 \pm 7\%$ respectively, $p=0.11$), however, T3 tumors had a significantly worse survival compared to both T1 ($p < 0.001$) and T2 ($p < 0.001$) tumors. Five-year survival was significantly worse in T3 tumors when compared to combined T1 and T2 tumors ($11 \pm 3\%$, $p<0.001$, Fig. 3.3). T4 tumors were associated with significantly worse survival when compared to T1 ($p < 0.001$), T2 ($p < 0.001$) and T3 ($p < 0.001$) tumors. LVI, tumor grade, type of surgical resection, age, gender and year of resection were not significant predictors of survival. There was a significant ($p < 0.001$) difference in 5-year survival between node negative and node positive tumors ($58 \pm 11\%$ vs. $9.8 \pm 4\%$ respectively; $p < 0.001$, Fig. 3.5).

Chi-square test (Table 3.6) demonstrated a strong association between T status and LVI ($p < 0.001$), T status and N status ($p < 0.001$) and N status and LVI ($p < 0.001$). A subgroup analysis was conducted to explore the relationship between T status and LVI in a subset of node negative patients.

3.6.4 Node Negative Subgroup Analysis

At least 15 lymph nodes were resected in 199 (34%) of 577 cases, 94 (47%) of the 199 were node-negative (Table 3.1). Among the node-negative patients, 56 (60%) were male and 38 (40%) female, with a mean age of 74 years (range 41 – 97 years). The median tumor size was 4.0 cm (range 0.5 – 13.0 cm). Lymphovascular invasion (LVI) was absent in 59 (63%), present in 22 (23%) and not recorded in 13 (14%) cases.

In a subgroup of node-negative patients, Cox's regression showed T status ($p<0.001$) and LVI ($p = 0.03$) to be independent predictors of survival. With T4 tumors removed ($n = 6$), due to small sample size, re-analysis showed only LVI (HR = 2.42; 95% CI 1.06 – 5.53) to be an independent predictor of disease-specific survival. T stage, gender, histological classification, tumor grade, esophageal and duodenal margin status, type of surgical resection, year of surgery, age, tumor size and residual tumor status had no influence on long-term survival.

There was a significant difference in the disease-specific 5-year survival in the presence or absence of LVI (34 $\pm$ 20% vs. 65 $\pm$ 14% respectively, $p = 0.016$, Fig. 3.6). A significant difference was found between T stage (T1-T3) and LVI ($p = 0.003$). The 5-year survival for T1 (64 $\pm$ 17%) and T2 (53 $\pm$ 14%) was not significantly different ($p = 0.11$); however, it was significantly worse for both T3 (11 $\pm$ 5%, $p < 0.001$) and T4 (1 $\pm$ 1%, $p < 0.001$) tumors. After stratifying by negative LVI, there was no significant difference between T stages ($p = 0.33$, Fig. 3.7).

The mean number of resected lymph nodes in the node-negative subgroup was 8.1 nodes (range 1 – 30). Seventy-six (80.9%) of 94 patients had less than 15 lymph nodes resected, while 16 (17.0%) had greater than 15 lymph nodes resected. There was no significant difference in 5-year disease-specific survival in the < 15 node group when compared to the > 15 node group, despite the notable difference in 5-year survival (57.3 $\pm$ 12.4% vs. 71.1 $\pm$ 24%, $p = 0.31$, Fig. 3.8).

3.7 Discussion

The Dutch gastric cancer and the British MRC trials, while attempting to settle the controversy with regard to appropriate lymphadenectomy, demonstrated the difficulty, even under the most controlled conditions, with compliance when performing standardized surgical procedures.[61, 63] This difficulty has been a point of contention with

respect to survival differences observed from specialized versus non-specialized centers, where the effects of stage migration have impaired study comparability.[3] Studies from specialized western centers applying standardized extended (D2) resections have supported reports from Asian centers[65, 72, 106]; demonstrating extended resections may be conducted safely with improved long-term survival compared with limited resections.[55, 62, 66, 70, 71, 75] These studies have confirmed lymph node status as the most important predictor of long-term survival. Despite these results, standardization and compliance with extended resections continues to be a problem.[13, 76, 78] Although specialized centers have been successful in achieving adequate lymph node resections (> 15 nodes)[62, 66, 75], the same cannot be said of non-specialized institutions. Mullaney et al.[76] demonstrated that 31% of surgically resected United Kingdom cases could be accurately staged as described, while Hundahl et al.[13] showed only 18% of US cases resected could be adequately staged for lymph node involvement.

This study examined the biologic predictors of long-term survival in a population-based cohort with the aim of establishing generalizability with European population-based studies and to determine if the results obtained from specialized western institutions also apply to non-specialized centers. In addition, potential surrogate predictors of survival that may improve prognostication when faced with the issue of inadequate lymph node staging were examined.

Our results showed 34% of cases in an unselected population had an adequate lymph node resection. This was consistent with European population studies, which in a similar time period, reported adequate lymph node resections of 23.2%[192] and 25.5%[194]. We report a 5-year disease-specific survival of 28%. This is in agreement with several European studies[9, 193, 194] that reported disease-specific 5-year survivals of 20-30.6% in unselected patients operated on with curative intent.

Our results confirmed that N status and T status were the most important independent predictors of long-term survival, followed by M status, histological classification and residual tumor status, consistent with published results obtained from both specialized and community-based centers.[65, 66, 75, 192, 194], and provide a basis for ongoing validity testing and generalizability of results among North American centers. Although we document the same independent predictors of survival, both the percent of

patients receiving an adequate node resection and the disease-specific survival differed markedly from those reported by specialized centers.[65, 66, 75, 197] This discrepancy would support the importance of instituting standardized extended lymph node resections in non-specialized centers to achieve optimum survival rates, similar to centers with dedicated gastric cancer resection protocols. However, the majority of North American and European centers does not perform adequate lymph node resections, and are unlikely to formally adopt this practice in the near future.[13, 76, 192] Because the ability to accurately predict outcome following surgery is limited when faced with incomplete staging information, additional markers of biologic behavior should be explored for their predictive ability.

Although LVI was significant by univariate analysis, it was not significant in a multivariate model. We believe that LVI loses its predictive ability in a population model due to the overwhelming significance of lymph node metastasis and deeply penetrating tumors (advanced N and T status). The fact that node status has the greatest impact on survival may merely reflect an end-stage in the natural progression of gastric cancer. We hypothesize that in early gastric cancer, T status and LVI are the most important determinants of subsequent nodal involvement. In keeping with this concept, we found a high correlation between advancing T and N status and the presence of LVI. This finding prompted us to examine a subgroup of node-negative patients to further evaluate the relationship of LVI with T and N status. Our findings are consistent with several studies[110, 112, 113], in which in a subgroup of node-negative patients, T status and LVI were found to be independent predictors of long-term survival. Previous studies suggest that vascular invasion may be an indicator of biologic aggressiveness independent of T status.[112] After excluding T4 tumors, we found that LVI alone emerged as an independent predictor of long-term survival ($p = 0.03$). When T4 tumors were included in the model, both LVI ($p = 0.04$) and T status ($p < 0.001$) retained predictive significance, however, given the small number of T4 tumors, a larger study is needed to fully establish the importance of T4 tumors in association with LVI. Overall, the documentation of LVI provides an additional source of information in predicting long-term survival. In non-specialized centers where many patients offered surgical therapy may not have an

adequate node resection (> 15 nodes), the combined use of LVI and T status may be used to improve prognostication.

Two separate studies [75, 78] previously reported a threshold value above which the number of resected nodes no longer significantly raised the proportion of tumors classified as node positive. These studies suggest that staging is reliable when at least 10 lymph nodes are removed and assessed by a pathologist. We propose that the addition of LVI to T status and N status might improve staging and prognostication. With increasing experience with at least 10 lymph nodes resected, the addition of factors such as LVI may allow for more accurate prognostication, thereby reducing the requirement for an extended resection, with its attendant increase in morbidity and mortality outside of specialized units.

Our results provide a population-based validation of independent predictors of long-term survival in patients with gastric cancer, and support the importance of standardizing surgical approaches to gastric cancer if population-based survival rates are to equal those of specialized oncology centers. In addition, we showed that LVI is highly correlated with advancing T and N status and is an independent predictor of survival in a subgroup of node-negative gastric cancer. We suggest that LVI, when combined with available lymph node data, may improve prognostication when lymph node stage is questionable. Future studies examining the significance of LVI will provide important insight into the role of LVI as a potential surrogate to lymph node staging.

Table 3.1 Baseline and operative characteristics of study cohort and subgroup of node-negative patients.

	Population n = 577 (%)	Node-Negative Subgroup n = 94 (%)
Mean Age	72.6	73.3
Gender		
Male	344 (60.0)	56 (60.0)
Female	233 (40.0)	38 (40.0)
Adjuvant Therapy		
Chemo.	42 (7.3)	2 (2.1)
Radio.	52 (9.0)	1 (1.1)
Operative Procedure		
Total	95 (16.5)	23 (24.5)
Subtotal	219 (38.0)	71 (75.5)
Palliative	29 (5.0)	-
Laparotomy	51 (8.8)	-
Esophageal Resection Margin		
Negative	280 (48.5)	90 (95.7)
Positive	34 (5.9)	4 (4.3)
Unresectable	178 (30.8)	-
Missing	85 (14.7)	-
Duodenal Resection Margin		
Negative	293 (50.8)	91 (96.8)
Positive	21 (3.6)	3 (3.2)
Unresectable	179 (31)	-
Missing	84 (14.6)	-
Hospital Mortality	57 (9.9)	3 (3.2)
Survival Status		
Alive	81 (14.0)	54 (57.4)
Dead	492 (85.3)	40 (42.6)
Missing	4 (0.7)	-

Chemo. - Adjuvant or neoadjuvant chemotherapy, alone or in combination.
Radio. - Adjuvant or neoadjuvant radiotherapy, alone or in combination.
Total - total gastrectomy.
Subtotal - subtotal gastrectomy.
Palliative - surgical intervention without resection or resection without curative intent.
Laparotomy – surgical intervention with discovery of inoperable disease.

Table 3.2 Tumor Characteristics of study cohort and subgroup of node-negative patients.

	Population n = 577 (%)	Node-Negative Subgroup n = 94 (%)
T stage		
T1	37 (6.5)	28 (30.0)
T2	57 (10.0)	29 (31.0)
T3	202 (35.0)	31 (33.0)
T4	101 (17.5)	6 (6.0)
X	164 (28.0)	-
Missing	16 (3.0)	-
N Stage		
N0	94 (16.0)	94 (100)
N1	183 (32.0)	-
N2	89 (15.0)	-
N3	16 (3.0)	-
X	141 (24.0)	-
Missing	54 (9.0)	-
M Stage		
M0	125 (22.0)	94 (100)
M1	216 (37.0)	-
Mx	236 (41.0)	-
***Stage**		
IA	30 (5.0)	28 (30.0)
IB	37 (6.0)	29 (31.0)
II	54 (9.0)	31 (33.0)
IIIA	106 (18.0)	6 (6.0)
IIIB	40 (7.0)	-
IV	236 (41.0)	-
X	73 (13.0)	-
‡Size (cm)		
<3.5	107 (18.5)	39 (41.0)
3.6-5.0	86 (15.0)	20 (21.0)
5.1-8.5	72 (12.5)	18 (19.0)
>8.6	86 (15.0)	9 (10.0)
Missing	226 (39.0)	8 (9.0)
†Tumor Histology		
Adenocarcinoma	311 (54.0)	46 (49.0)
Intestinal	52 (9.0)	14 (15.0)
Diffuse	53 (9.0)	9 (10.0)
Signet cell	90 (16.0)	21 (22.0)
Mucinous	18 (3.0)	2 (2.0)
Undifferentiated	53 (9.0)	2 (2.0)

Table 3.2 Continued.

Lymphovascular Invasion		
Absent	92 (16.0)	59 (63.0)
Present	250 (43.0)	22 (23.0)
X	147 (26.0)	-
Missing	88 (15.0)	13 (14.0)
Grade		
Low	21 (4.0)	6 (6.0)
Mod	125 (22.0)	33 (35.0)
High	385 (67.0)	48 (51.0)
Missing	46 (8.0)	7 (7.0)
Residual Tumor Status		
R0	231 (40.0)	87 (93.0)
R1/R2	80 (13.0)	7 (7.0)
X	183 (32.0)	-
Missing	83 (14.0)	-

X - Unresectable tumor.

*Stage - as described by the AJCC/UICC 5th edition.[19]

‡Size - measured in greatest transverse diameter (cm).

†Tumor histology as proposed by the World Health Organization histological classification of gastric tumors.[19]

Table 3.3 Disease-specific 5-year survival by AJCC/UICC stage in the study cohort and the node-negative subgroup of patients

	n (%)	5-year survival %	Median survival years
Population cohort (n = 577)			
***Stage**			
IA	30 (5.2)	60.5	6.1
IB	37 (6.4)	60.1	6.3
II	54 (9.3)	55.2	6.2
IIIA	106 (18.4)	11.2	1.0
IIIB	40 (6.9)	5.2	0.83
IV	236 (40.9)	4.3	0.25
X	73 (12.6)	1.4	0.30
Node-Negative Subgroup (n = 94)			
***Stage**			
IA	28 (29.7)	67.9	6.5
IB	29 (30.8)	57.9	5.7
II	31 (32.9)	60.6	6.2
IIIA	6 (6.4)	16.7	0.72
IV	-	-	-
X	-	-	-

X denotes unresectable tumor.

* Stage – recorded as described by the AJCC/UICC 5th edition as follows:

	N0	**N1**	**N2**	**N3**
T1	IA	IB	II	IV
T2	IB	II	IIIA	IV
T3	II	IIIA	IIIB	IV
T4	IIIA	IV	IV	IV

Hayashi et al.[198]

Table 3.4 Population-based model: Independent univariate predictors of long-term survival (n = 577)

Covariate	Wald statistic	df	p-value
T status	253.3	4	< 0.001
N status	194.7	4	< 0.001
M status	238.4	1	< 0.001
TNM Stage	282.1	6	< 0.001
Tumor Histology	29.3	5	< 0.001
Lymphovascular Invasion	150.4	2	< 0.001
Tumor Grade	16.3	2	< 0.001
Duodenal Margin Status	212.6	2	< 0.001
Esophageal Margin Status	214.8	2	< 0.001
Type of Surgery	241.1	4	< 0.001
Tumor Size	32.7	4	< 0.001
Year of Surgery	1.4	1	0.97
Age	5.3	4	0.26
Gender	0.5	1	0.47

Cox's univariate regression significant at $p < 0.05$

Table 3.5 Population-based final model: Independent multivariate predictors of long-term survival (n = 577)

Covariate	β	SE	HR	95% CI	p-value[a]
T status					**< 0.001**
T1			1	-	
T2	0.11	0.39	1.12	0.52 – 2.41	
T3	0.90	0.36	2.46*	1.22 – 4.98	
T4	1.50	0.42	4.38**	1.92 – 9.95	
X	0.88	0.65	2.42	0.68 – 8.61	
N status					**< 0.001**
N0			1	-	
N1	0.81	0.21	2.25**	1.50 – 3.38	
N2	1.25	0.24	3.48**	2.16 – 5.60	
N3	1.30	0.53	3.67*	1.29 – 10.42	
X	-0.01	0.47	0.99	0.39 – 2.50	
M status					**0.033**
M0			1	-	
M1	0.46	0.21	1.57*	1.04 – 2.39	
Tumor Histology					**0.002**
Adenoca.			1	-	
Intestinal	0.70	0.21	1.07	0.71 – 1.61	
Diffuse	0.39	0.23	1.48	0.94 – 2.33	
Mucinous	-0.81	0.44	0.44	0.19 – 1.06	
Signet Cell	-0.43	0.22	0.65	0.42 – 1.00	
Undiff.	-1.10	0.40	0.33**	0.15 – 0.71	
Esophageal Margin					**0.014**
Negative			1	-	
Positive	-0.22	0.36	0.80	0.39 – 1.63	
X	1.25	0.54	3.50*	1.22 – 9.98	
‡Tumor Size					**0.089**
< 3.5			1	-	
3.6-5.0	-0.07	0.20	0.93	0.62 – 1.39	
5.1-8.5	0.41	0.21	1.50*	1.00 – 2.25	
> 8.51	0.17	0.20	1.19	0.79 – 1.77	

β – Denotes coefficient; a – overall p-value for covariate; *p <0.05; **p < 0.01

‡Tumor size found to be confounding variable with clinical importance and included in final model.

Undiff. – Undifferentiated tumor histology as defined by WHO histological classification

AdenoCa – Adenocarcinoma as defined by WHO histological classification

X – inclusion of patients with missing values; if missing variable coded as 99 in analysis

Table 3.6 Association between tumor thickness, nodal involvement and lymphovascular invasion.

	T status	N status	LVI
T status	-	< 0.001*	< 0.001*
N status	-	-	< 0.001*
LVI	-	-	-

* Chi-square p-value.

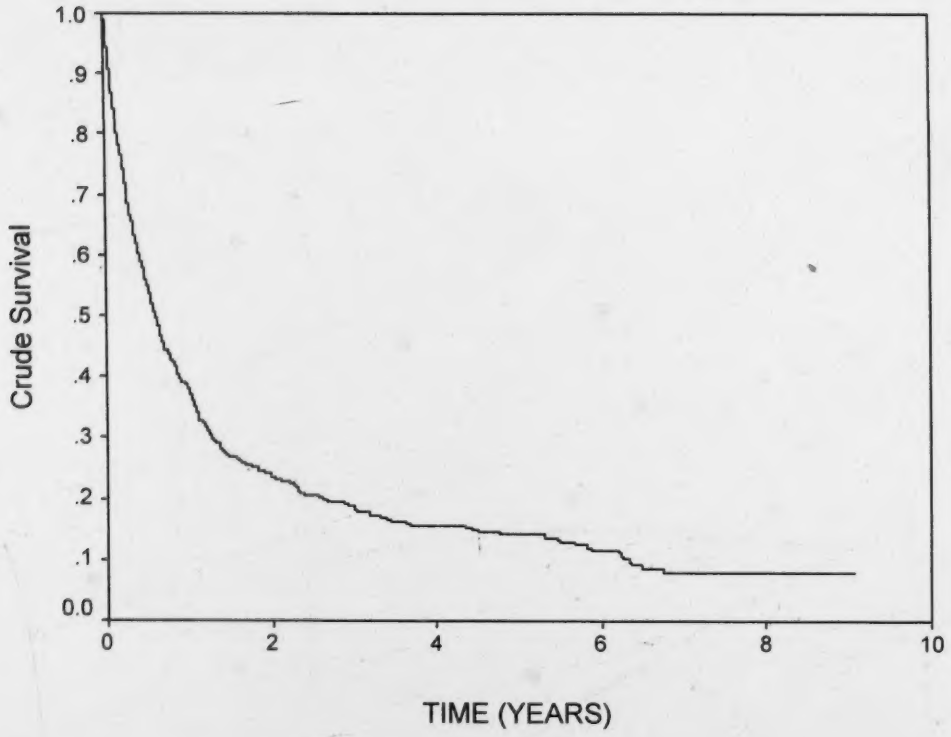

Figure 3.1 Crude overall survival for the entire study population (n = 577). The 5-year crude survival rate is 12% (95% CI: 8.9 – 14.9). The 5-year disease-specific survival rate for the entire study population is 28% (95% CI: 21.9 – 33.2).

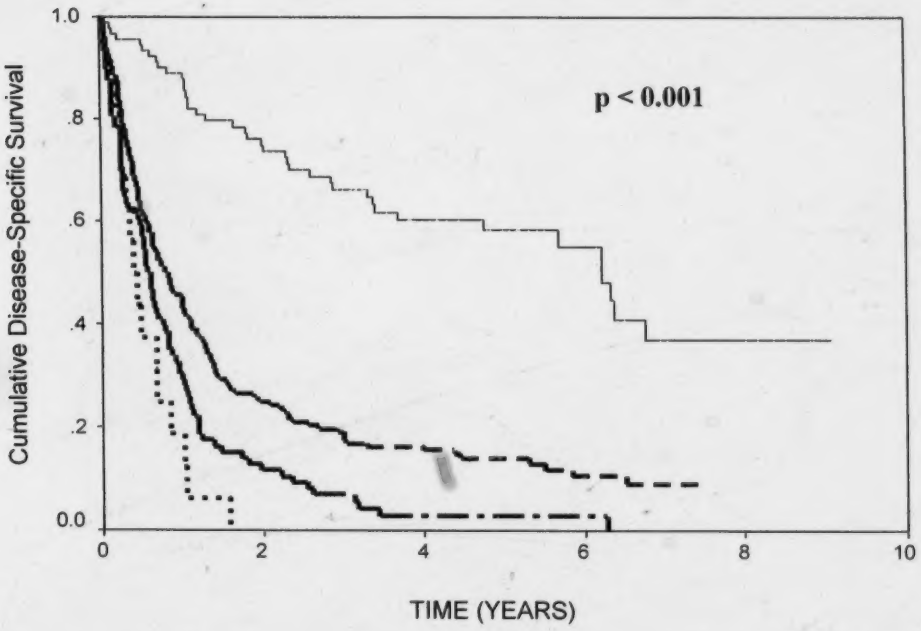

Figure 3.2 Kaplan-Meier curve of disease-specific survival by node status. N0 = 0 nodes; N1 = 1-6 nodes; N2 = 7-15 nodes; N3 = > 15 nodes involved respectively. Unresectable tumors (n = 140) are not shown. There was a significant difference in long-term survival between node positive and node negative tumors (9.8 ± 4% vs. 58 ± 11% respectively).

Legend: Node Status

N0 (n = 94)

N1 (n = 183)

N2 (n = 88)

N3 (n = 16)

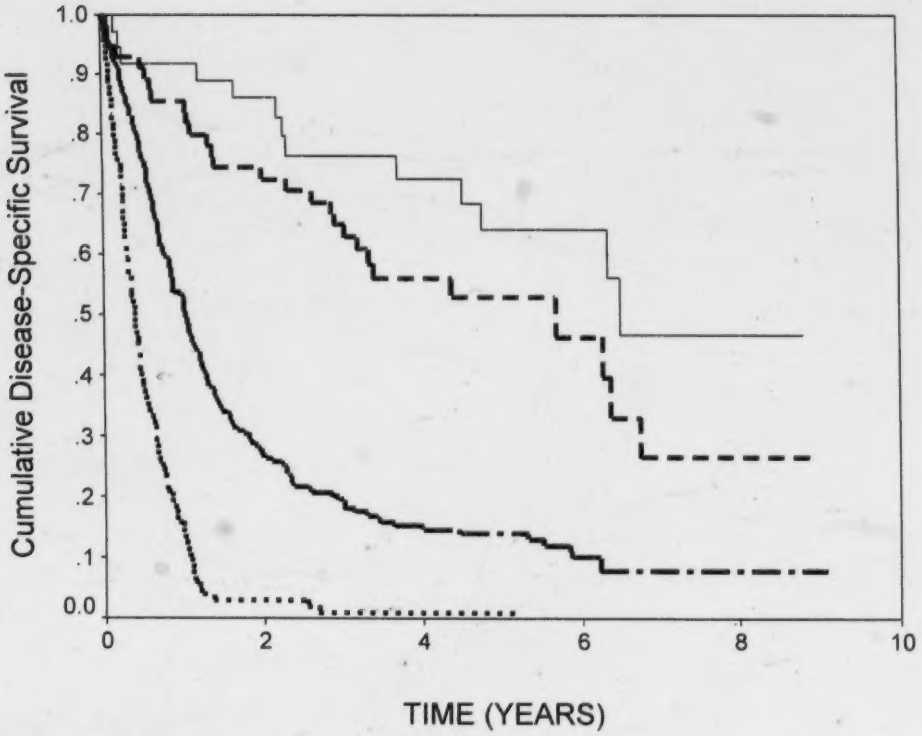

Figure 3.3 Kaplan-Meier curves for disease-specific survival by tumor thickness (T status). Unresectable tumors (n = 162) are not shown. There was no significant difference between T1 and T2 tumors ($p = 0.11$). When combined, there was a significant difference between superficial tumors (T1 and T2) and both T3 ($p < 0.001$) and T4 tumors ($p < 0.001$).

Legend: Tumor Thickness

T1 (n = 37)

T2 (n = 57)

T3 (n = 202)

T4 (n = 101)

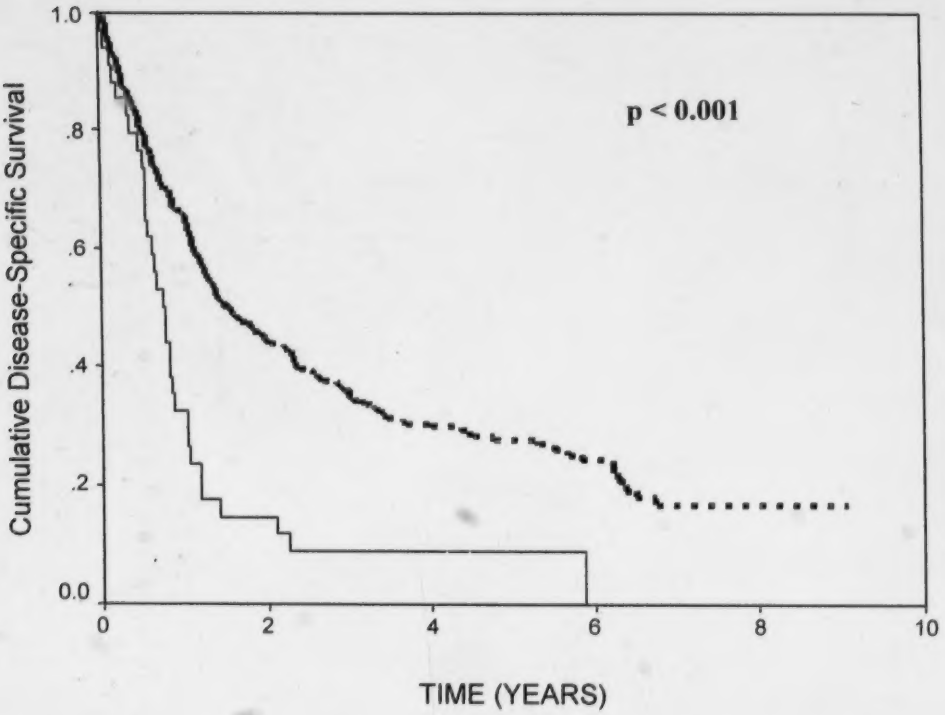

Figure 3.4 Kaplan-Meier curve of disease-specific survival by esophageal resection status. Negative status denotes the absence of viable tumor cells within 1 cm of the resection margin. Positive status denotes the presence of tumor cells at the resection margin. Unresectable tumors (n =176) are not shown. Positive esophageal margins were associated with significantly worse survival compared with negative resection margins (8.8 $\pm$ 3% vs. 27.5 $\pm$ 5%, respectively).

Legend: Esophageal Margin

.....
Negative (n = 280)

Positive (n = 34)

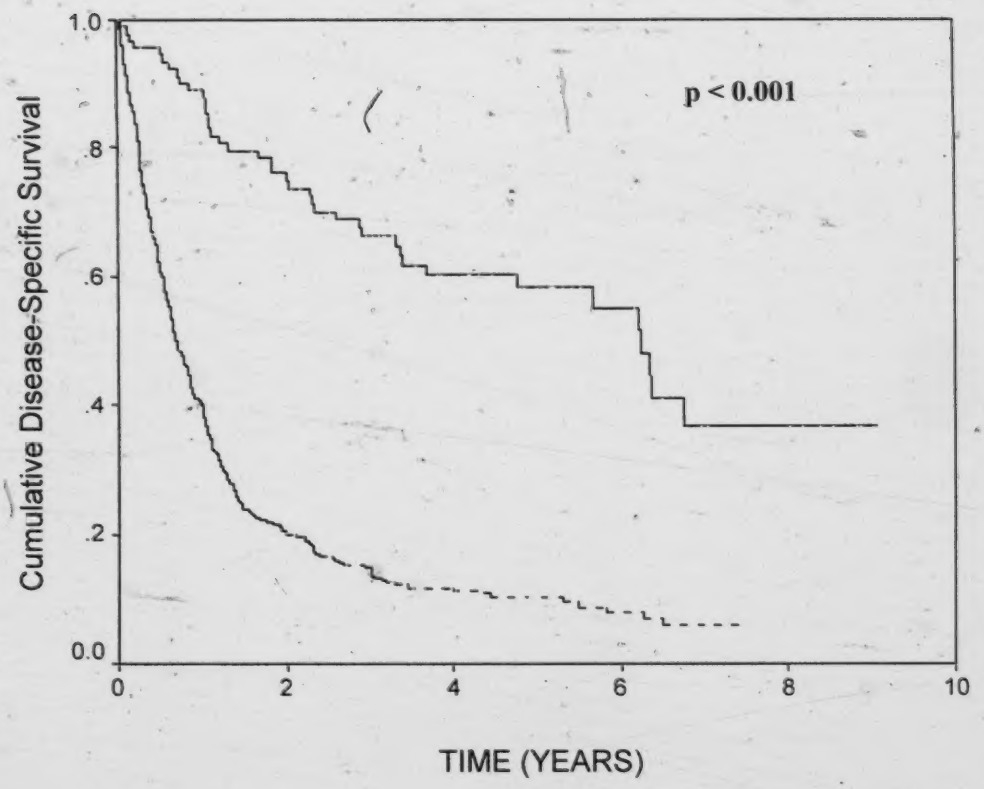

Figure 3.5 Kaplan-Meier curve of disease-specific survival stratified by lymph node status. There is a significant difference in disease-specific 5-year survival between node positive and node negative patients (9.8 ± 4% vs. 58 ± 11% respectively). Unresectable tumors (n = 141) and cases with missing lymph node status (n = 54) are not shown.

Legend: Node Status

Node negative
(n = 94)

Node positive
(n = 279)

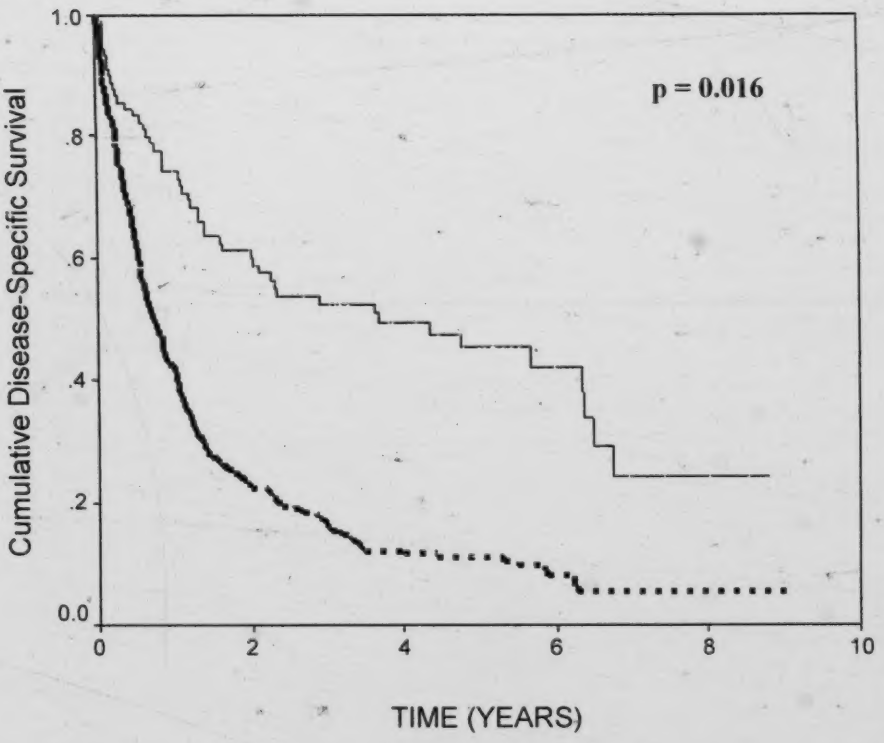

Figure 3.6 Kaplan-Meier curves for disease-specific survival by lymphovascular invasion status in the node-negative subgroup. Unresectable tumors (n = 145) are not shown. The presence of LVI positive tumors was associated with significantly worse survival compared to LVI negative tumors (34 ± 20% vs. 65 ± 14% respectively).

Legend: Lymphovascular Invasion (LVI)

LVI negative
(n = 92)

.....
LVI positive
(n = 250)

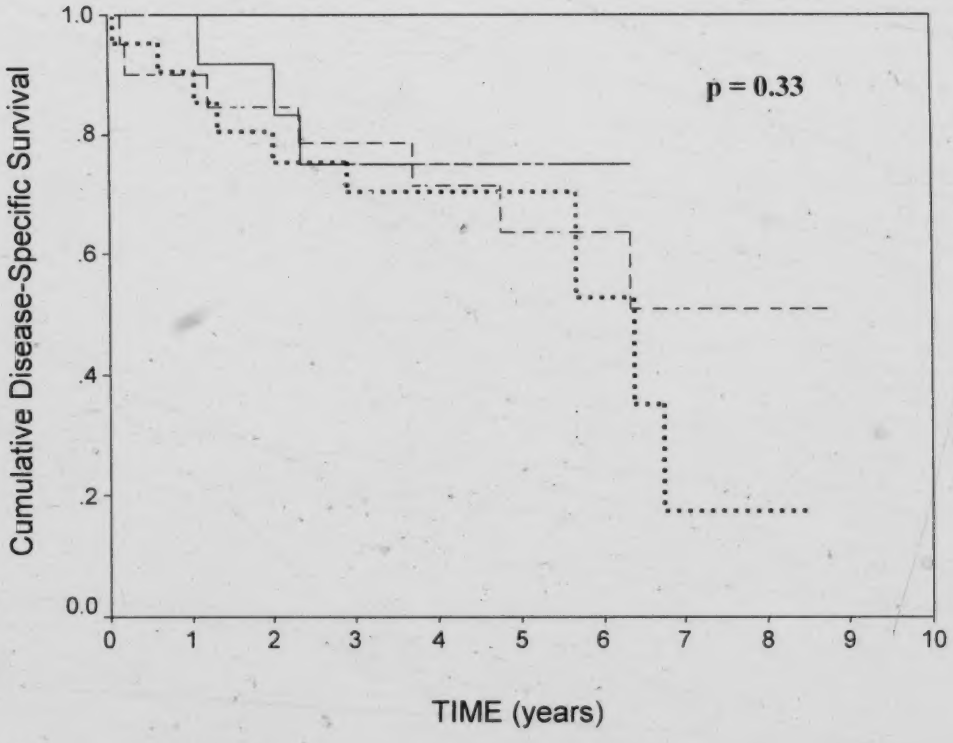

Figure 3.7 Kaplan-Meier curves of disease-specific survival for tumor thickness (T status) in a lymphovascular negative and node-negative subgroup of patients (n = 94). There was no significant difference between T1 and T2 (p = 0.51), T1 and T3 (p = 0.59) and between T2 and T3 (p = 0.61). T4 tumors (n = 6) were excluded due to small sample size. When T3 and T4 were combined there was no difference between T1 (p = 0.66) and T2 (p = 0.65) tumors.

Legend : T status

T1 (n = 20)

T2 (n = 22)

T3 (n = 14)

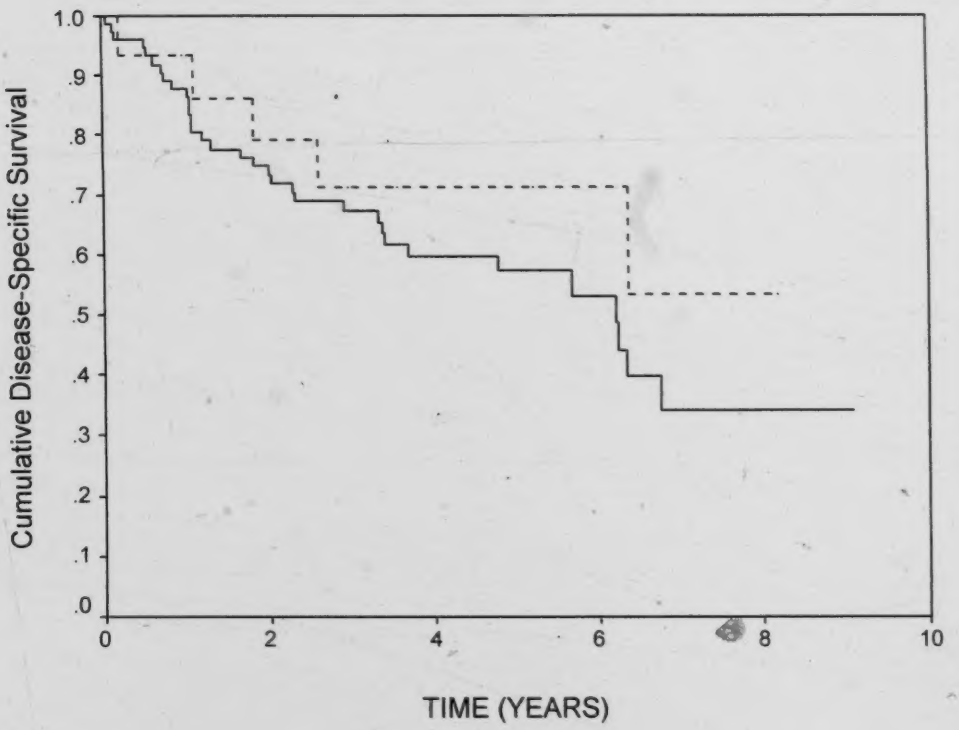

Figure 3.8 Kaplan-Meier curves of disease-specific survival in a subgroup of node-negative patients comparing < 15 lymph node resection to > 15 lymph node resection. No significant difference in survival in the < 15 node group compared to the > 15 node group (57.3 + 12.4% vs. 71.1 + 24.0% respectively, p = 0.31).

Legend: N status

> 15 nodes
(n = 16)

_____
< 15 nodes
(n = 76)

Chapter Four

Prospective Gastric Cancer Model

4.1 Introduction

Surgical resection for gastric cancer continues to be the primary modality, with complete locoregional control being the only chance for cure. [9, 68] However, even after potentially curative surgery, up to 80% of patients will develop tumor recurrence. [199] This is compounded by the observation that 65% of gastric cancers in the United States present at an advanced stage, with nearly 85% of tumors accompanied by lymph node metastasis at diagnosis.[13] The incidence of nodal involvement has given rise to controversy regarding what is considered to be an appropriate lymphadenectomy. It is unlikely however that the issue of lymphadenectomy will be settled in the absence of more specific markers of biologic behavior, which may be used to improve prognostication and provide targets for improved management strategies.

Lymphovascular invasion (LVI) predicts poor outcome in several malignancies, including gastric cancer.[178, 199-202] In a recent review[203], LVI emerged as a prognostically promising factor, which independently predicted survival and was associated with advanced T stage, prompting some authors to suggest that LVI should be included in risk stratification and selection of patients for entry into clinical trials.[178, 199] In a follow-up study, our results indicated that LVI was predictive of poorer survival in node-negative patients selected from a population-based cohort, [204] and were in agreement with previous studies examining node-negative gastric cancer, further supporting LVI as a potential marker of biologic behavior. [110, 112, 113] To better understand the role of LVI in gastric cancer, complete delineation of the pathways preceding lymphatic permeation is necessary.

Cyclooxygenase-1 (COX-1) is a constitutively active enzyme, involved in maintaining normal tissue homeostasis, including cytoprotection of the gastric mucosa.[167] Constitutive expression of COX-1 in gastric tissue provides a useful control in protein localization studies. Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins.[205] COX-2 is an inducible gene-product whose expression is enhanced by stimuli such as inflammation, cytokines, tumor promoters and growth factors.[169, 205, 206] Studies have shown that increased levels of COX-

2 favor malignant growth in many tumor types, including gastric cancer, by giving tumor cells a survival advantage through inhibition of apoptosis and immune surveillance and promotion of angiogenesis. [167] [168] COX-2 levels correlate with lymphatic permeation, tumor thickness and lymph node metastasis. [167, 169, 170, 206] Recent studies have shown that the signaling protein, integrin-linked kinase (ILK), once stimulated, is capable of inducing expression of invasion-related genes such as COX-2, which is believed to stimulate activation of the matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9), thereby facilitating tumor invasion through degradation of the basement membrane, and allowing access to the lymphatic and vascular spaces.[171, 176, 207, 208]

We conducted a study in a population-based cohort with gastric cancer in which independent predictors of survival and the presence of COX-1, COX-2, MMP-2 and MMP-9 immunoreactivity were considered for their abilities to predict biologic behavior with respect to T status and LVI. We also conducted gene expression analysis in gastric tumors using oligonucleotide microarray analysis to identify the genetic determinants of gastric cancer behavior.

4.2 Objectives and Hypotheses

The primary objective of this portion of the study was to assess whether the addition of protein immunoreactivity improves the prediction of cause-specific mortality in gastric cancer patients outcome compared to standard histological and pathological staging criteria. In addition, prospectively gathered gastric cancer specimens were processed by DNA microarray methods to examine potential predictive gene expression profiles with respect to LVI. The specific objectives were both descriptively and analytically based. This study involves several steps:

1. Construction of multi-tumor tissue array blocks for protein immunoreactivity studies.
2. Assessment of clinicopathologic factors and immunoreactivity profiles for cause-specific mortality and pathological characteristics in a retrospective cohort of gastric cancer patients using multivariate modeling.
3. Construction of a prospective gastric cancer tumor bank and DNA microarray studies to examine the potential predictive profiles with respect to LVI.

4.2.1 Descriptive Objectives

i) To describe the distribution of clinicopathologic variables in a retrospective cohort of patients with resected primary gastric adenocarcinoma (n = 114).

ii) To describe the distribution of clinicopathologic variables in a prospective cohort of patients presenting with primary gastric adenocarcinoma (n = 20).

4.2.2 Analytic and Methodologic Objectives

i) Determine prognostic factors for disease-specific survival in a cohort of patients with resected gastric cancer through the application of multivariate analyses.

ii) Determine the relationship between protein immunoreactivity profiles and clinicopathologic factors and disease-specific survival.

iii) Conduct oligonucleotide microarray studies of freshly banked gastric cancer to examine potential predictive gene expression profiles with respect to LVI.

4.2.3 *A Priori* Hypotheses

The following hypotheses were established *a priori*:

i) Immunoreactivity of the protein markers cyclooxygenase-1 (COX-1), cyclooxygenase – 2 (COX-2), metalloproteinase 9 (MMP-9) and metalloproteinase 2 (MMP-2) correlate with tumor thickness (T status) and lymphovascular invasion (LVI) and can be demonstrated through immunohistochemical analysis of archival gastric cancer tissue (n = 114).

ii) Tumor thickness (T1/2 vs. T3/4) and lymphovascular invasion (LVI positive vs. negative) are important prognostic factors.

iii) LVI status can be predicted by gene expression profiles.

4.2.4 Research Hypotheses

i) The application of immunoreactivity studies to multivariate analyses improves the prediction of outcome when compared to standard histological and pathological staging criteria alone.

ii) Gene-expression profiles are capable of predicting tumor behavior with respect to LVI.

4.3 Relevance of the Study

There is a paucity of literature that has attempted to incorporate multivariate analyses with microarray-based predictive gene profiles. This technology has been established in other types of cancer with encouraging results. These studies have demonstrated that gene expression profiles are capable of out-performing standard pathologic and clinical criteria in predicting outcomes. If the application of gene expression profiles demonstrates improved classification and predictive abilities, it would seem justified to begin to include a "molecular-based" staging system into the present pathologic-based system. The ability to obtain mucosal biopsies from patients during endoscopic investigations has allowed researchers the opportunity to diagnose early gastric cancer and confirm malignancy in patients presenting with symptoms. This technique however is limited in its ability to provide information beyond a histological diagnosis. Subsequent management of patients has relied primarily on intraoperative findings and pathological staging. However, Kuwahara et al.[209] have demonstrated the ability to extract RNA from endoscopically obtained gastric mucosal biopsies of pre-malignant and malignant tissue. The ability to extract and amplify RNA with available methods may allow clinicians to utilize gene expression profiles to predict the biological behavior of a tumor pre-operatively. This would allow a tailored surgical approach, thereby minimizing unnecessary and potentially morbid procedures or by optimizing extensive resections in patients most likely to benefit from aggressive surgical therapy.

STEP ONE:

4.4 Materials and Methods

4.4.1 Study Design

This step involves multivariate modeling of a retrospective cohort (n = 114) of patients with resected gastric cancer. This population of patients was selected from a population-based database, as described in section 3.3.2, over the period of January 1, 1994 through December 31, 1997. Inclusion required a diagnosis of primary gastric adenocarcinoma based upon histologic classification (WHO criteria), complete clinicopathologic data and either a gastric cancer specimen or formalin-fixed paraffin archival block for pathologic review. Demographic, clinicopathologic, operative and

outcome data was obtained from the population-based gastric cancer database as described (sections 3.3.4 – 3.3.7).

4.4.2 Archival Gastric Tumor Samples

All surgical specimens undergo formalin fixation, gross pathological examination and sampling of both normal and malignant tissue for the creation of permanent paraffin-embedded tissue blocks for microscopic slide preparation. In Edmonton, tissue blocks are stored in archival libraries at the UAH and DKML according to the hospital where the initial surgery took place. A search of the central pathology archive database, using the keyword "gastric adenocarcinoma" identified all patients with pathological specimens. The search was limited to those patients having had a surgical resection of the primary tumor. This excluded endoscopically obtained mucosal biopsies, peritoneal biopsies, and metastatic gastric deposits to adjacent organs, peritoneal cytology washes and resection of the omentum. These criteria generated 114 archival cases corresponding to Group I participants. A cross reference of the pathology synoptic report with the Group I database was conducted to ensure all archival specimens had representative clinicopathological information catalogued in the database. An anatomical pathologist compiled a list of tissue blocks from the pathology reports for each patient case. Histology slides corresponding to the tissue blocks were assembled for each patient. The UAH provided 37 patient cases with corresponding histology slides and tissue blocks (n = 110). DKML provided the remaining 83 patient cases (345 tissue blocks). Corresponding slides (n = 345) from DKML were newly cut by the University of Alberta Medical Laboratory Services.

Histology slides (n = 455) were used to confirm the presence, the location and the orientation of tumor elements within the tissue blocks and the block most representative of the primary gastric tumor from each case for tissue sampling. From the 455 slide and block pairs, 114 patient cases were selected for tissue array construction. Patient confidentiality was maintained through the use of unique pathology accession numbers cross referenced to a patient list under the direction of the primary investigator.

4.4.3 Instrumentation and Multi-Tumor Tissue Array Construction

H & E-stained histology slides were used to define tumor regions. Slides with representative malignant epithelial elements were selected, marked with a grease pen and

oriented with the corresponding tissue block to facilitate micro-core (1.0 mm x 3.0 mm) biopsy. A recipient block was created using a tissue arrayer (Beecher Instruments, Silver Springs, MD). The process involved two punch biopsies: one to create an array hole in the recipient paraffin block and one to collect donor gastric tissue from the original archival block. Selection of donor tissue was facilitated by aligning a freshly cut pre-marked H&E slide corresponding to the donor block. A recipient block was prepared by melting regular paraffin and pouring into a standard tissue block mold. One millimeter (1.0 mm) donor tissue biopsies were placed at 1.0 mm intervals with the assistance of the X-Y precision guide rails of the Beecher instrument. The recipient block was oriented from left to right.

Prior to sectioning, the recipient block surface was smoothed and leveled by warming the block to promote adherence of the biopsies to the array block holes. While warm, the tissue biopsies are leveled by applying gentle pressure with a smooth surface, thereby pushing the tissue cores to the same level. Tissue sections containing the arrayed samples may be cut from the recipient block using standard microtome techniques.

4.4.4 Immunohistochemical Analysis

Four-μm sections of the resulting multi-tumor tissue array block were transferred to glass slides. The sections were deparaffinized in xylene, re-hydrated in graded ethyl alcohol (100% X3, 80%, 70%, 50% then water), and then washed in running tap water. The slides were antigen retrieved in a TRIS (pH 10.0) retrieval solution (DAKO cat # S3307) under pressure and heat at 100°C for 10 minutes. The slides were sequentially cooled, washed in running water for 10 minutes and incubated in 3% H2O2 and methanol to deplete endogenous peroxidase activity. Finally, the slides were washed in running water for 10 minutes then placed in phosphate buffered saline. The multi-tumor tissue arrays were immunostained with anti-mouse antibodies (Novocastra Laboratories Ltd, Newcastle, UK) by the avidin-biotin peroxidase complex method. Monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA) and included MMP-2 (monoclonal IgG_1, 2C1), MMP-9, (monoclonal IgG_1, 2C3), COX-1 (monoclonal IgG_{2b}) and COX-2 (monoclonal IgG). Tissue arrays were scored independently by two pathologists (SA and RL) blinded to the clinical outcome of the patients, employing a semi-quantitative scoring system. For each antibody studied, location of

immunoreactivity (cytoplasmic, nuclear or combined) was noted, and staining intensity was graded from 0 (no staining) to 2 (strong staining).

4.4.5 Statistical Methods

All methods employed are as previously described (section 3.5), with the exception of a logistic regression outlined below. In brief, the gastric cancer database analyses were undertaken with SPSS statistical software, version 11.0 (Chicago, IL). Patient- and tumor-factors were entered as categorical variables. Continuous variables were assessed for linearity and, where appropriate, transformed into categorical variables. Survival curves were calculated using the Kaplan-Meier method, then compared using the log-rank test. The effect of patient-factors, tumor-factors and immunoreactivity of protein markers with disease-specific survival was assessed through a Cox's proportional hazard model, applying a purposeful selection method. [196] The significance of the covariates was tested using the Wald test. No variables included in the final model violated the proportional hazard assumption. The association between T status and LVI with protein-expression profiles was tested using a Chi-square. A p-value < 0.05 was considered statistically significant.

4.5 Results – Step One

4.5.1 Clinical and Operative Characteristics

The clinicopathologic characteristics of the retrospective cohort ($n = 114$) are shown in Table 4.1. The prospective DNA microarray subgroup ($n = 20$) is shown in Table 4.1 for the purpose of comparison. With respect to the retrospective population, the majority of patients (68.4%) presented with locally advanced gastric cancer, where 70 (61.4%) had T3 and 8 (7.0%) had T4 tumors (Table 4.1). The small number of T4 tumors was related to the exclusion of unresectable tumors, where archival tissue blocks were not available for protein-expression studies. LVI was significantly associated with both T status ($p = 0.001$, Figure 4.1) and N status ($p < 0.001$, Figure 4.1). LVI was reported in 68 (59.6%), absent in 37 (32.5%) and not reported in 9 (7.9%) cases. The presence of LVI was associated with a significantly worse 5-year survival compared with LVI negative tumors ($13.9 \pm 8.4\%$ vs. $55.9 \pm 16.6\%$ respectively, $p < 0.001$, Fig. 4.2). Of the patients with LVI, there where 2 (2.9%) T1 tumors, 10 (14.7%) T2 tumors, 49 (72.1%) T3 tumors and 7 (10.3%) were T4 tumors. At least 15 lymph nodes were resected in only 10 (8.8%)

resected cases, while 33 (28.9%) had less than 15 lymph nodes removed and 71 (62.3%) could not be accurately staged according to the AJCC staging criteria (5th Edition, Appendix 2).

4.5.2 Population-Based Outcomes

The median follow-up was 19.2 months (range 1 – 120). At the time of analysis, 26 (22.8%) patients were alive and 88 (77.2%) were dead. There were 6 (5.3%) in-hospital mortalities not related to gastric cancer. The 5-year disease-specific survival was 29.8% (95% CI 21.6 – 38.4).

Table 4.2 shows the results of the univariate and multivariate Cox's proportional hazard model. In the univariate analysis T, N, M, LVI, esophageal margins, tumor size, R status, COX-1 and MMP-2 expression were significant predictors of outcome. In contrast, gender, age, histologic type, tumor grade, type of surgical resection and over-expression of COX-2 and MMP-9 were not significant prognostic factors.

By multivariate analyses, M status was the most significant independent prognostic factor ($p < 0.001$), followed by LVI ($p = 0.013$, Fig. 4.2) and N status ($p = 0.033$). MMP-2, although not an independent predictor of survival, was of borderline significance ($p = 0.053$) and included in the final model. T status, esophageal margin status, tumor size, R status and protein-expression profiles were not significantly associated with disease-specific survival (Table 4.2).

4.5.3 Protein Immunoreactivity

A representative color figure of a multi-tumor gastric cancer tissue array is shown in Figure 4.3. COX-2 immunostaining was localized predominantly in the cytoplasm of gastric cancer cells (Figure 4.4B) and was not detectable within the tumor stroma. In contrast, COX-1 immunostaining was more variable, demonstrating cytoplasmic and/or nuclear localization. The presence of COX-1 and COX-2 immunoreactivity was demonstrated in 64.1% and 82.5% of gastric cancer specimens, respectively. COX-1 immunoreactivity correlated significantly with tumor grade ($p = 0.003$), where 32 (80%) of 40 poorly differentiated tumors failed to express COX-1. COX-2 immunoreactivity significantly correlated with T status ($p = 0.02$) and tumor grade ($p = 0.01$). Although COX-2 immunoreactivity was not significant with respect to N status, there was a significant association when nodal involvement was stratified into positive versus

negative ($p = 0.02$, Table 4.3) tumor involvement. COX-2 immunoreactivity was significantly associated with both MMP-2 ($p < 0.001$) and MMP-9 ($p < 0.001$) expression.

Of 114 cases studied, MMP-2 immunoreactivity was not expressed in 29 (25.4%), weak in 56 (49.1%) and strong in 29 (25.4%) patients. MMP-2 immunoreactivity was not associated with any clinicopathologic factors examined (Table 4.3). MMP-9 immunostaining was localized primarily to the cytoplasm of gastric cancer cells (Figure 4.5B) with little to no stromal staining. Of 114 gastric cancer samples studied, 56 (49.1%) had weak staining, 26 (22.8%) had strong staining and 32 (28.1%) demonstrated no immunostaining (Figure 4.5A). MMP-9 immunoreactivity exhibited borderline significance with both T status ($p = 0.07$) and lymph node positivity ($p = 0.08$).

There was a significant association in the pattern of immunoreactivity between MMP-2 and MMP-9 ($p < 0.001$), where there was concordance in the immunoreactivity in 64.5% of tumors with weak staining, 63% with moderate staining and 48.3% among tumors staining strongly with MMP-2 and MMP-9.

STEP TWO

4.6 Materials and Methods

4.6.1 Study Design

This was a prospective cohort design. Patients diagnosed with gastric cancer or referred to four Edmonton hospitals (University of Alberta, Royal Alexandra, Grey Nuns and Misericordia) with a diagnosis of gastric cancer from January 1, 2002 to December 30, 2003 inclusive were eligible for inclusion (Group II). Follow-up on the prospective cohort continued until February 15, 2004. All Group II patients gave informed consent to allow data collection of their clinicopathological data, collection of a peripheral blood sample and collection of a fresh tumor sample from the resected gastric tumor. Collection of blood and tumor samples, in addition to the immunohistochemistry (IHC) constitutes the molecular component of this study, and is referred to hereafter as the "molecular data". All molecular analyses were conducted prospectively.

4.6.2 Selection of Cohort

Patients included in this study were those patients admitted to one of the four Edmonton hospitals with a diagnosis of primary gastric adenocarcinoma between January 1, 2002 and December 30, 2003. Patients were identified through direct communication with the responsible surgeon following initial diagnosis or referral. To ensure complete capture of patients, a cross-check was undertaken each week during accrual by checking the operative slate at each hospital for any cases booked for distal esophagectomy, gastrectomy (subtotal or total) or palliative bypass for malignancy. Definitive cases were identified prior to surgery based upon histological diagnosis from endoscopic investigations. Histologic criteria followed the WHO criteria.

4.6.3 Inclusion and Exclusion Criteria

Group II patients' medical, clinical, pathological and surgical information were collected prospectively. The inclusion and exclusion criteria applied to the prospective cohort are as outlined in section 3.3.3.

Thirty-six patients initially diagnosed as having gastric carcinoma were subsequently excluded for the following reasons: 11 (19.6%) cases, despite adequate tumor harvesting, failed to produce sufficient total RNA for microarray analysis, 4 (7.1%) lymphoma, 4 (7.1%) GIST, 12 (21.4%) found to be unresectable at surgery and 5 (8.9%) refused consent. The resulting 20 patients constituted the microarray gene-expression cohort, and were entered into a prospective database. Only those patients having a successful resection of the primary tumor (curative or palliative) were included in the molecular analysis.

4.6.4 Clinicopathologic Sources of Data

Clinicopathologic data was entered prospectively into a database for Group II patients (n = 20). Survival status was entered as the date of death or the end of patient accrual. Cross validation of clinicopathologic and outcome variables was conducted through the CCI cancer registry and vital statistics as with Group I. Clinicopathological data was ascertained on all patients regardless of surgical therapy; however, tumor samples were collected only on patients having had a resection.

4.6.5 Cross Cancer Institute Records

The Cancer Registry provided updated survival status and was used to verify demographic data for Group II patients. Survival status was ascertained up to February 15, 2004.

4.6.6 Surgeon Communication

Prior to commencement of enrolment of Group II patients, all 28 surgeons practicing in the four Edmonton hospitals were contacted for their assistance in recruiting patients undergoing surgical therapy for primary gastric cancer. In addition, the pre-admission clinics and surgical patient-care coordinators at each site were contacted at the initiation of the study and weekly thereafter for complete patient accrual. An information letter included a brief description of the objectives, methodology and a copy of the ethics approval. Information regarding surgical therapy or the extent of disease that was not provided in the final pathology synoptic report was ascertained through direct communication with the responsible surgeon.

4.6.7 Fresh Tumor Specimens and Tumor Banking

Patients identified for possible surgical resection of a gastric cancer provided informed consent pre-operatively. Personal communication between the primary investigator and responsible surgeon ensured tissue collection and adherence to collection protocol.

Following extirpation, the surgical specimen was taken immediately to a staging area, where within 20 minutes of devitalization; at least 1.0 mL of fresh tumor was sampled from the gastric tumor by an anatomical pathologist. Individual tumor samples were deposited into a 5 mL eppendorf tube, labeled and snap-frozen in liquid nitrogen. Extra samples were taken if the tumor was large and additional sampling would not jeopardize pathological characterization. In addition, grossly normal gastric mucosa was harvested from a site away from the primary tumor to be used in microarray studies. All eppendorf tubes were labeled with a central catalogue number as well as marked to differentiate normal mucosa from tumor. Catalogue numbers acted as the only tissue identifiers and maintained patient confidentiality. The tumor type, location, special characteristics, number of samples taken and the devitalization time and storage time were recorded with corresponding central catalogue number. The primary investigator

cross-referenced the final pathology report diagnoses with the central catalogue numbers. Non-adenocarcinoma diagnoses were excluded from subsequent studies.

All tissue harvesting was conducted by an anatomical pathologist or senior pathology resident (SA) to ensure sampling of viable tumor, sampling of normal mucosa and to avoid compromising the surgical specimen for subsequent pathological characterization. Where tumor was judged to be too small to allow both tumor sampling and pathological studies, sampling was deferred. Similarly, where tissue devitalization time exceeded 20 minutes, tumor samples were not taken. Liquid nitrogen storage containers were present on site at each of the four Edmonton hospitals where tissue samples were stored until transfer to the CCI. Samples were transferred to the CCI on a weekly basis and stored in a freezer.

4.6.8 Patient Blood Samples

The collection of patient blood samples was coordinated with medical laboratory services at each hospital to take place at the time of routine pre-operative blood testing to minimize patient discomfort. An Alberta Cancer Board (ACB) polyomx research blood requisition was created to meet the requirements of medical laboratory services (Appendices 6 and 7). Blood tubes were labeled with a central catalogue number in a fashion identical to the collection of fresh tumor specimen. Blood samples were collected and stored for future analysis.

4.6.9 DNA Microarray - Tissue RNA Preparation and Processing

Snap-frozen gastric tumor (n = 20) is removed from the storage eppendorf tube, cut into small cubes, homogenized in a Trizol (1 mL/100mg) solution (Life Technologies, Inc.), and then centrifuged at 3700 rpm for 25 minutes. The resultant lysate is treated with a 70% ethanol, applied to an RNeasy column (Qiagen) and centrifuged at 3700 rpm for 5 minutes. The RNA is subsequently eluted from the column into a fresh tube with 0.8 mL of RNase-free water centrifuged at 5000 rpm for 3 minutes. Following isolation, a quality control check is carried out by running 1 *u*g of eluted RNA in an ethidium bromide gel. The remaining RNA is stored as an ethanol precipitate in liquid nitrogen. Isolation of RNA from normal mucosa for reference standards is undertaken in an identical manner. A reference sample was generated from one part normal gastric mucosa added to an equal amount of total RNA prepared from 17 pooled gastric cancers.

Isolated mRNA (10 *u*g aliquots) from each tumor is used for cDNA synthesis by reverse transcription (Superscript II; Life Technologies, Inc.). Three tumor total-RNA reactions for each patient are pooled to generate a total of 30 *u*g of cDNA. The same process is applied for reference cDNA synthesis. Pooled cDNA is cleaned (QIAquick PCR Purification Kit, Qiagen) over a column, washed, and then precipitated with 70% ethanol. The cDNA is re-suspended in sodium bicarbonate (0.1 M) to which Cy3 (9 *u*l) and Cy5 (9 *u*l) is added to the tumor and reference cDNA respectively. The labeled samples are then washed over a purification column (QIAquick kit) and precipitated.

4.6.10 Microarray Slide Preparation and Hybridization

Microarray slides are prepared with sequential 0.1% SDS, ddH_2O, and alcohol wipes. In preparation for slide construction, the cDNA pellets are re-suspended in ddH_2O (5 *u*l). A hybridization solution in then prepared in an eppendorf tube containing reference cDNA (5 *u*l), tumor cDNA (5 *u*l), GFP-Cy3 (1 *u*l), salmon sperm (2 *u*l), tRNA (2 *u*l), and Easy Hybridization solution (45 *u*l). The probe is next denatured in a PCR machine, cooled, and loaded onto the glass slide support (50 *u*l) and covered overnight at 42˚C. The hybridized slides were washed prior to scanning.

4.6.11 Microarray Slide Scanning

Fluorescent images of the hybridized microarray slides are obtained using a microarray scanner (Axon Instruments, Foster City, CA). Fluorescent images are scanned at 532 nm (green) and 635 nm (red). Primary data collection and data analysis of the images generated are carried out with GenePix Pro 3.0 (Axon Instruments). The raw data is stored for statistical analysis in a gastric cancer microarray database.

4.6.12 Statistical Methods - Gene-Expression Analysis

Analysis of microarray data was performed using the Nearest Shrunken Centroid (NSC) method.[210] Each clinical parameter of interest was divided into two groups to match the clinically important groups as closely as possible: T1 and T2 versus T3 and T4 were used as binary classifiers. LVI negative versus LVI positive was similarly used as binary classifiers. A binary classifier was built using the specified classes with one clinical parameter at a time. Cross-validation over various Δ values was used to choose an optimal Δ, a parameter in the NSC method which indirectly sets the number of genes used in the classifier and needs to be empirically determined. Leave-one-out cross-validation

was conducted. Optimal Δ implies maximal accuracy and a minimum number of genes. Baseline accuracy is measured using a 'majority classifier', where each instance is assigned the label of the majority class: the larger the class imbalance, the greater the baseline accuracy.

4.7 Ethical Considerations

Ethical approval for the assembly of archival gastric cancer tissue from the University of Alberta pathology archives and DKML was provided by the HERB (Appendix 8). As this involved review and analysis of discarded tissue, no informed consent was required by the Ethics committee. Patients enrolled in the prospective cohort involved chart reviews, collection of fresh gastric cancer tissue and blood collection. A copy of the ethics approval is shown in Appendix 9. Informed consent was obtained on all patients presenting with presumed gastric cancer (Appendix 10).

4.8 Results –Step Two

From January 1, 2002 to November 30, 2003 inclusive, patients newly diagnosed with primary gastric adenocarcinoma, meeting the inclusion criteria, were consented and enrolled into the prospective arm of this study. During the accrual period 20 patients with a diagnosis of gastric adenocarcinoma were identified, and consented to allow collection of clinicopathologic, operative, tumor-related and outcome data. All 20 patients consented to the collection of fresh gastric cancer tumor specimen for tumor banking and subsequent DNA microarray analysis and peripheral blood sampling for future genomic analysis related to gastric cancer research.

4.8.1 Clinical and Operative Characteristics

The clinical and operative characteristics of the prospective cohort (n = 20) are presented in Table 4.4. There were 15 (75.0%) males and 5 (25.0%) females with a mean age of 73.5 years (range 53.0 – 88.0 years). In the microarray cohort, 5 (25.0%) patients had a total gastrectomy and 15 (75.0%) had a subtotal gastrectomy (Table 4.1). Esophageal margins were microscopically negative in 19 (95.0%) and positive in 1 (5.0%).

4.8.2 Tumor Characteristics

Tumor characteristics of the microarray cohort are presented in Table 4.1. Tumors were most commonly observed penetrating the serosa (T3) in the microarray cohort of

patients. Tumor involving at least 6 lymph nodes (N1) was the most common presentation in the microarray cohort (40.0%). However, it is important to note that 53.6% of patients had less than the minimum 15 lymph nodes to accurately stage the nodal status. Overall, 28.6% of the microarray cohort of patients presented with advanced stage IV disease. All patients had tumor specimen for assessment of LVI status. Five cases (25.0%) were LVI negative and 15 cases (75.0%) were LVI positive (Table 4.1).

4.8.3 Prospective Gastric Cancer Cohort Outcomes

Median follow-up was 1.7 years. Seventeen (85.0%) patients were alive and 3 (15.0%) were dead. All recorded deaths were gastric cancer related. To date no patients have been lost to follow-up. Short follow-up time in the prospective arm of this study limits any meaningful survival analysis. Long-term follow-up with ongoing patient accrual will facilitate subsequent analyses in the future.

4.9 Gene-Expression Analyses

The clinicopathologic characteristics of the microarray cohort are shown in Table 4.4. Gene-expression analysis was undertaken to determine if patterns in the gene expression profile relate to the T status or LVI status of the samples studied. No patients in the microarray cohort had T1 tumors, whereas there were eight (40.0%) T2 tumors, ten (50%) T3 tumors and two (10.0%) patients with T4 tumors. For the purpose of analysis the tumors were grouped into T1/T2 and T3/T4. LVI was present in 15 (75.0%) and absent in 5 (25.0%) tumors analyzed.

The gene-expression profile and reported gene functions of the genes whose class means differed most between the two classes (T1/T2 relative to T3/T4) are shown in Table 4.5. Microarray gene-expression analysis was unable to demonstrate a profile biologically predictive of local tumor invasion when examined with respect to superficial versus deeply penetrating tumors (T1/T2 versus T3/T4), despite a significant association demonstrated between increasing depth of tumor invasion and overall survival when examined by multivariate Cox's regression (Table 3.5 & Figure 3.3). More specifically, when grouped into superficial (T1/T2) versus deeply penetrating tumors (T3/T4), there was no difference in the genetic profile that would allow biologic characterization based upon tumor thickness.

The gene-expression profile and reported gene functions of the genes whose class means differed most between classes with respect to LVI are presented in Table 4.6. There were six genes whose class means had the greatest expression in the LVI positive relative to the LVI negative. These included genes associated with proteolysis, G-protein-modulation, cell-adhesion and cell migration. Four genes whose class means had the least expression in the LVI positive relative to the LVI negative included genes associated with kinase inhibition, cell-cycle control, signal transduction, oncogenesis and protease inhibition. Of these genes, most interesting, was ribophorin II, which is associated with a glycosyl transferase involved in T-cell activation. Despite the observation that LVI could independently predict poor overall survival by multivariate analyses (Figure 3.6); it was not possible to construct a classifier that could reliably predict LVI; this may have been due to the small sample size.

4.10 DISCUSSION

This study examined the clinicopathologic predictors of survival in a population-based cohort, and correlated these factors with protein-expression profiles. In addition, oligonucleotide microarray analyses were conducted to look for gene expression patterns that could be correlated with clinical parameters such as LVI.

The incidence of LVI in gastric cancer varies from 5.4% to 86%, with the lowest incidence reported in patients with node-negative tumors.[112, 211] In our analysis, 59.6% of patients resected for cure were found to have evidence of LVI. LVI has previously been reported to be an independent risk factor for long-term survival [212] and for the risk of lymph node metastasis.[213] It has been suggested that LVI may be a clinically useful marker of biologic aggressiveness.[110] This observation was subsequently supported by Hyung et al.[112] who demonstrated that LVI was an adverse prognostic indicator independent of clinicopathologic factors in node-negative gastric cancer. This study concluded that LVI may provide useful information for prognosis and clinical management in the subset of patients with node-negative gastric cancer.[112] More recently, Kooby et al.[113] showed that in node-negative patients, vascular invasion was an independent predictor of poor outcome and identified more aggressive lesions independent of tumor size and depth of invasion. This finding was consistent with our

earlier results, which in a subgroup analysis demonstrated that LVI was independently associated with poor outcome in node-negative gastric cancer.[204]

Our earlier studies differed from the present one in that we included all patients with gastric cancer resected for cure, regardless of nodal status. By multivariate analysis, our results indicated that N status ($p = 0.033$), M status ($p < 0.001$) and LVI ($p = 0.013$) were independent predictors of survival in patients with resected gastric cancer. This observation is supported by Talamonti et al.[199] who recently showed that, along with other clinicopathologic factors, LVI was an independent predictor of disease-free survival. The latter study reported five-year overall survival rates of 26.2% in LVI positive compared to 49.9% in LVI negative tumors. In the present study, we demonstrated a strong correlation between LVI and T status ($p = 0.001$, Fig. 2) and N status ($p < 0.001$). Furthermore, we showed that patients with LVI had significantly worse five-year survival compared to LVI negative tumors ($13.9 \pm 8.4\%$ vs. $55.9 \pm 16.7\%$ respectively, $p < 0.001$, Fig. 4.1).

Examining protein immunoreactivity provides an opportunity to correlate observed clinicopathologic characteristics with specific gene products governing tumor behavior. The present study demonstrated a significant association between COX-2 immunoreactivity and gastric cancer with respect to depth of tumor invasion, tumor grade and the presence of lymph node involvement. Tumor grade [167, 168] and depth of tumor invasion[167-169, 214] have been shown to correlate with COX-2 expression in gastric cancer. It has been proposed that the activity of COX-2 is facilitated through enhanced activity of the matrix metalloproteinases.[206] In the present study we showed a significant association between COX-2 and MMP-2 ($p < 0.001$) or MMP-9 immunoreactivity ($p < 0.001$), which was consistent with the results of gene transfection studies that showed that COX-2 expression increased the metastatic potential of colon cancer through activation of the MMP-2.[215] It has recently been demonstrated that COX-2 and the MMPs are up-regulated following mitogenic stimulation of phosphoinositide 3-kinase (PI3K).[207, 216] Activated PI3K is believed to act through a pleckstrin homology domain (PH) of an integrin-linked kinase (ILK) with subsequent activation of protein kinase B (PKB/Akt).[208] Once activated, PKB/Akt up-regulates the transcriptional factor NF-kB.[208, 217] Importantly, MMP-9 has been shown to be expressed in a NF-kB-dependent manner.[208, 217] The

observed association between COX-2 and MMP-9 may therefore suggest a role of COX-2 in the activation of MMP-9 as previously suggested.[208, 217]

We did not show an association between COX-1, COX-2, MMP-2 or MMP-9 immunoreactivity and LVI. MMP-9 however demonstrated borderline significance with both lymph node positivity and depth of tumor invasion, as previously shown.[171, 174] Overall, this pattern of expression was consistent with the concept that both COX-2 and MMP-9 may function at an early stage in gastric cancer, thereby giving invasive cells a survival advantage through early access to the lymphatic and vascular spaces, facilitated through degradation of the basement membrane.[171, 173, 174] Furthermore, there was a significant concordance between MMP-2 and MMP-9 immunoreactivity, perhaps suggesting co-dependence in the process involved with tumor penetration and lymphovascular invasion.

Oligophrenin-I (OPHN1) is a GTPase-activating protein that has been shown to stimulate GTP hydrolysis of signaling intermediates such as Rac1.[218, 219] Once activated, these intermediates regulate functions such as cell-cell and cell-matrix adhesion, membrane trafficking and transcriptional regulation.[219] Recently, OPHN1 was identified within the enteric plexus, where it was hypothesized to be involved with gastrointestinal disease and recovery after injury.[218] Similarly, Pinheiro et al.[220], using cDNA microarray analysis, demonstrated over-expression of OPHN1 in colorectal tumors. In the present study we documented differential expression of the gene encoding OPHN1 in gastric cancer tissue. Although the exact role of OPHN1 in gastric cancer is unknown, previous studies have shown the ability of OPHN1 to activate Rac1 [221]. Rac1, following stimulation by phosphoinositide 3-kinase (PI3K), activates the Akt/PKB intracellular pathway mediating cellular migration and invasion through MMP-9 modulation.[217] Interestingly, OPHN1 contains a pleckstrin homology domain (PH), and some PH domains bind PI3K products with high affinity.[222] The differential expression of the OPHN1 gene in colorectal cancer[220], gastric cancer[223] and in the present study provide interesting insight into the behavior of gastric cancer, and more importantly this observation appears to agree with our clinical and protein immunoreactivity analyses.

The late events of T-cell activation are believed to be associated with N-linked glycosylation, mediated by an oligosaccharyltransferase (OT),[224] which is a protein

complex consisting of ribophorin I, ribophorin II and a 50-kDa protein.[224] OT activity has been show to increase 10-fold during cytotoxic T-cell activation, and tumor-infiltrating lymphocytes produced up to 20-fold more glycoprotein than resting lymphocytes when stimulated by OT.[224] Since the majority of tumors express major histocompatibility complex type I (MHC-I), they are susceptible to destruction by activated T-cells,[225] which therefore play a key role in immune surveillance and anti-tumor activity in many human malignancies, including gastric cancer.[225-228] In the present study, when examining the class means of LVI positive relative to LVI negative gastric tumors, we documented a differential expression of ribophorin-II (RPN II), a protein that has not been previously documented in gastric cancer. Maehara et al.[227] suggested that when gastric cancer cells advance into the lymphatic space, an immunosuppressive activity is exerted and local defense mechanisms are suppressed. It is therefore of considerable interest that, in the present study, RPN II activity was differentially expressed on microarray analyses when examined with respect to LVI positive relative to LVI negative tumors.

In conclusion, our study demonstrated the significance of LVI as an independent predictor of survival in a population-based cohort of patients with gastric cancer. We document a significant association between COX-2 abundance and T status, tumor grade, lymph node positivity, and MMP-2 and MMP-9 abundance. Finally, we presented preliminary findings from gene-expression profiles of gastric cancer patients, which revealed expression of oligophrenin-I, a gene product potentially involved in mediating cellular migration and invasion of cancer cells through the basement membrane. In addition, we demonstrated the expression of ribophorin-II, a protein complex involved with T-cell activation, immune surveillance and potential anti-tumor activity. The identification of these gene products provides potential insight into the biology of LVI in gastric cancer. More detailed microarray profiles, employing larger sample sizes, in addition to tissue array technology may clarify the molecular alterations identified in this study.

Table 4.1: BASELINE CHARACTERISTICS OF THE RETROSPECTIVE AND PROSPECTIVE GASTRIC CANCER COHORTS

	[1]Retro Cohort n = 114 (%)	[2]Microarray Cohort n = 20 (%)
Age, yrs		
Mean	72.2	73.5
Range	28 – 98	53 - 88
Gender		
Male	65 (57.0)	15 (75.0)
T status		
T1	14 (12.3)	-
T2	22 (19.3)	8 (40.0)
T3	70 (61.4)	10 (50.0)
T4	8 (7.0)	2 (10.0)
X**	-	-
N status		
N0	40 (35.1)	6 (30.0)
N1	51 (44.7)	8 (40.0)
N2	20 (17.5)	6 (30.0)
N3	3 (2.6)	-
X**	-	-
M status		
M0	96 (84.2)	8 (40.0)
M1	18 (15.8)	5 (25.0)
Mx	-	7 (35.0)
***Tumor Histology**		
AdenoCa	60 (52.6)	15 (75.0)
Intestinal	17 (14.9)	3 (15.0)
Diffuse	12 (10.5)	1 (5.0)
Signet Cell	20 (17.5)	1 (5.0)
Undiff.	5 (4.4)	-
†Tumor Size (cm)		
< 3.5	32 (28.1)	4 (20.0)
3.6-5.0	27 (23.7)	4 (20.0)
5.1-8.5	30 (26.3)	9 (45.0)
>8.6	19 (16.7)	3 (15.0)
X**	6 (5.3)	-
Lymphovascular Invasion		
Absent	37 (32.5)	5 (25.0)
Present	68 (59.6)	15 (75.0)
X**	-	-
Missing	9 (7.9)	-

Grade		
Low	7 (6.1)	1 (5.0)
Mod	35 (30.7)	9 (45.0)
High	68 (59.6)	10 (50.0)
Missing	4 (3.5)	-
Residual Tumor Status		
R0	103 (90.4)	18 (90.0)
R1/R2	10 (8.8)	2 (10.0)
Missing	1 (0.9)	-
X	-	-
Number of Nodes Evaluated		
Mean	9.8	13.6
Median	8.0	13.0
Min	2.0	3.0
Max	31.0	29.0

[1]Retro = retrospective cohort, [2]Microarray = prospective DNA microarray cohort.
*Tumor Histology - World Health Organization histological classification of gastric tumors.[19]
**X – Unresectable Tumor.
†Tumor Size measured in greatest transverse diameter (cm).

Table 4.2 RETROSPECTIVE POPULATION-BASED UNIVARIATE AND MULTIVARIATE ANALYSIS (n = 114)

	Univariate			Multivariate		
Covariate	**HR**	**95% CI**	**p-value[a]**	**HR**	**95% CI**	**p-value[a]**
T status			**0.014**			
T1	1					
T2	0.85	0.37 – 1.94				
T3	1.81	0.92 – 3.54				
T4	2.98*	1.12 – 7.91				
N status			**< 0.001**			**0.033**
N0	1			1		
N1	2.97**	1.79 – 4.96		1.68	0.87 – 3.23	
N2	4.17**	2.25 – 7.72		2.87**	1.37 – 6.01	
N3	7.53**	2.21 – 25.65		3.01	0.75 – 12.92	
M status			**< 0.001**			**< 0.001**
M0	1			1		
M1	5.24**	3.04 – 9.04		4.39**	2.18 – 8.84	
Lymphovascular Invasion			**< 0.001**			**0.013**
Negative	1			1		
Positive	2.57**	1.56 – 4.25		2.13*	1.17 – 3.87	
Esophageal Margin Status			**0.004**			
Negative	1					
Positive	2.78**	1.39 – 5.57				
Tumor Size (cm)			**0.017**			
< 3.5	1					
3.6 – 5.0	1.28	0.69 – 2.37				
5.1 – 8.5	1.51	0.85 – 2.71				
> 8.6	2.65**	1.43 – 4.90				
Residual Tumor Status			**0.001**			
R0	1					
R1/R2	2.89**	1.52 – 5.47				
COX-1			**0.030**			
Absent	1					
Weak	1.59	0.99 – 2.55				
Strong	0.79	0.43 – 1.42				
MMP-2			**0.020**			**0.053†**
Absent	1			1		
Weak	2.17**	1.24 – 3.75		2.10*	1.14 – 3.87	
Strong	1.89*	1.02 – 3.51		1.56	0.79 – 3.06	

a – Overall p-value for covariate; *p < 0.05; **p < 0.01, †borderline significant.
Gender, tumor histology, grade, type of resection, age, COX-2 and MMP-9 were not significant by univariate analysis and are not shown.

Table 4.3 ASSOCIATIONS BETWEEN CLINICOPATHOLOGIC FACTORS AND IMMUNOREACTIVITY IN PRIMARY GASTRIC CANCER.

	COX-1	COX-2	MMP-2	MMP-9
LVI	0.70	0.46	0.41	0.21
T-status	0.20	0.02*	0.52	0.07†
Node positive	0.28	0.02*	0.18	0.08†
Tumor Grade	0.003*	0.01*	0.39	0.11

Numerical values represent Chi-square p-values, *statistically significance, †borderline significance.
LVI = Lymphovascular invasion
T status = Tumor thickness based upon TNM criteria.[19]
N positive = Node positive vs. Node negative
Tumor Grade = WHO histological classification.[19]

Table 4.4 OPERATIVE CHARACTERISTICS OF THE PROSPECTIVE GASTRIC CANCER COHORT.

	Gastric Cancer Cohort n = 20 (%)
Adjuvant therapy	
Chemotherapy	-
Radiotherapy	-
None	20 (100)
Operative Procedure	
Total Gastrectomy	5 (25.0)
Subtotal Gastrectomy	15 (75.0)
Laparotomy	-
X	-
Esophageal Resection Margins	
Negative	19 (95.0)
Positive	1 (5.0)
X	-
Duodenal Resection Margins	
Negative	18 (90.0)
Positive	2 (10.0)
X	-
Lymph Node Resection	
< 15 nodes	11 (55.0)
> 15 nodes	7 (35.0)
X	2 (10.0)
Perioperative Morality	
Yes	-
No	20 (100)
Survival Status	
Alive	17 (85.0)
Dead	3 (15.0)

Laparotomy – No surgical resection attempted - unresectable disease at time of surgery.
X – Unresectable tumor.

Table 4.5: LOCAL TUMOR INVASION (T STATUS) DIFFERENTIAL GENE-EXPRESSION PROFILE AND GENE FUNCTION

Rank	Description	Symbol	Function	Unigene[a]
1.	*KIAA0442 protein	AUTS2	Unclassified	32168
2.	*Ig Kappa constant region	-	Unknown Function	-
3.	*Calcyclin	S100A6	Signal Transduction, Calmodulin-related protein	275243
4.	†Lipase-gastric cancer	LIPF	Lipase, Hydrolase	159177
5.	*Ig Kappa variable 1D-8	-	Unknown Function	-
6.	*Hypothetical Protein	LOC90133	Unclassified	101651
7.	*Human putative FAP protein	-	Unknown Function	-
8.	*Ig lambda joining region	-	Unknown Function	-
9.	*High-mobility group	-	Unknown Function	-
10.	*Thymosin – Beta10	TMSB10	Unclassified	76293

Differential gene-expression profile of patients with primary gastric adenocarcinoma. The listed genes are those with the greatest differential expression relative to the pooled sample from 17 individuals. The genes are listed in rank order. Approximately 2000 genes were included on the microarray platform, with 938 genes included in the final analysis.

[a]Unigene identification number[229]

*Up-regulated gene-expression

†Down-regulated gene-expression

Table 4.6: LYMPHOVASCULAR INVASION GENE-EXPRESSION PROFILE AND GENE FUNCTION

Rank	Description	Symbol	Function	Unigene[a]
1.	*Human alpha satellite and Satellite-3 junction DNA sequence		Nuclear matrix/ scaffold protein binding	247946
2.	*Pepsinogen A	PGA5	Proteolysis	432854
3.	†GW128 Protein	YWHAB	Signal transduction/ Cell cycle control	279920
4.	*Oligophrenin 1	OPHN1	G-protein modulator Cell Adhesion	128824
5.	*CDC14	CDC14A	Phosphatase Protein phosphatase	65993
6.	†Junction Plakoglobin	JUP	Signal transduction Oncogene, cell adhesion	2340
7.	†Ribophorin II	RPN2	Glycosyl transferase T-cell activation	406532
8.	†C1 inhibitor	SERPING1	Protease inhibitor	151242
9.	*Kruppel-related Zinc finger	ZNF300	Transcription factor mRNA transcription	288928
10.	*Nucleolar autoantigen	SSA1	Zinc finger transcription factor	1042

Differential gene-expression profile of patients with primary gastric adenocarcinoma. The listed genes are those with the greatest differential expression relative to the pooled sample from 17 individuals. The genes are listed in rank order. Approximately 2000 genes were included on the microarray platform, with 938 genes included in the final analysis.

[a] Unigene identification number[229]

*Over-Expression

†Under-Expression

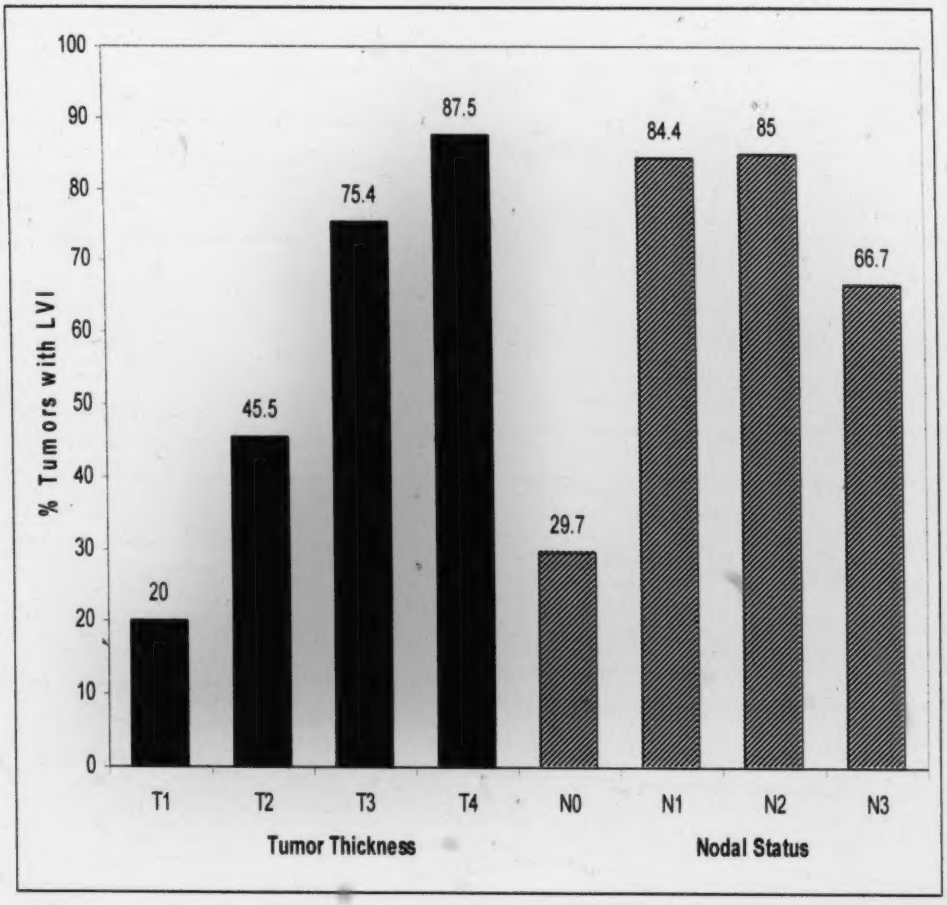

Figure 4.1: Relationship of lymphovascular invasion with tumor thickness and lymph node status. The chart demonstrates a significant association between advancing T status ($p = 0.001$) and advancing N status ($p < 0.001$) with LVI.

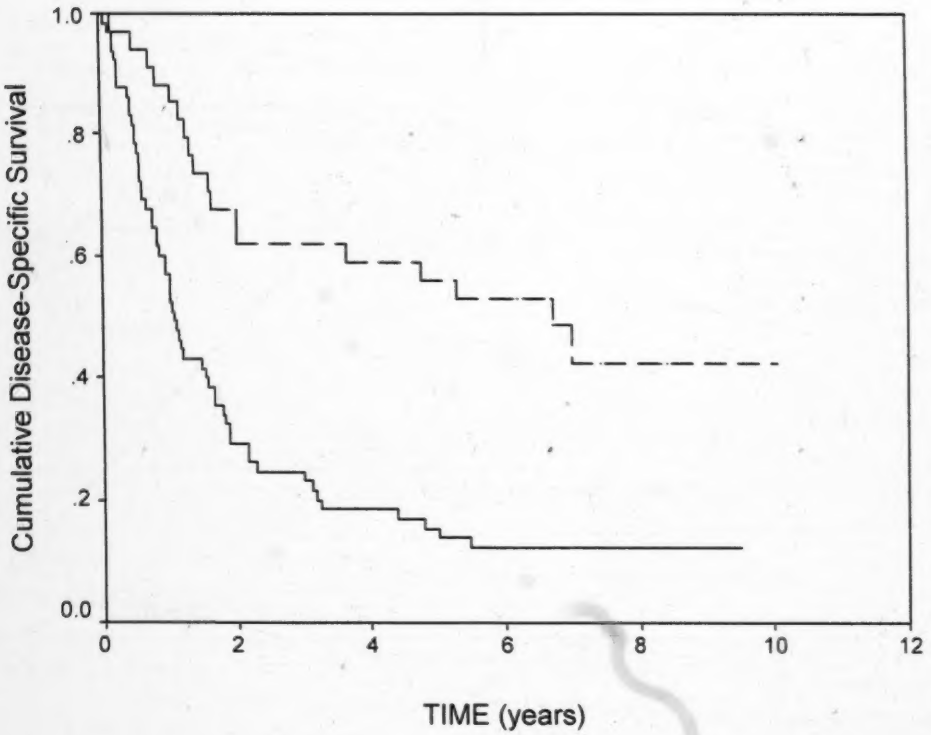

Figure 4.2: The relationship between LVI and disease-specific survival in a population of patients with resected gastric cancer. The presence of LVI was associated with a significantly worse 5-year survival compared to LVI negative patients (13.9 ± 8.4% vs. 55.9 ± 16.7% respectively).

Legend: LVI Status

Negative
(n = 34)

Positive
(n = 65)

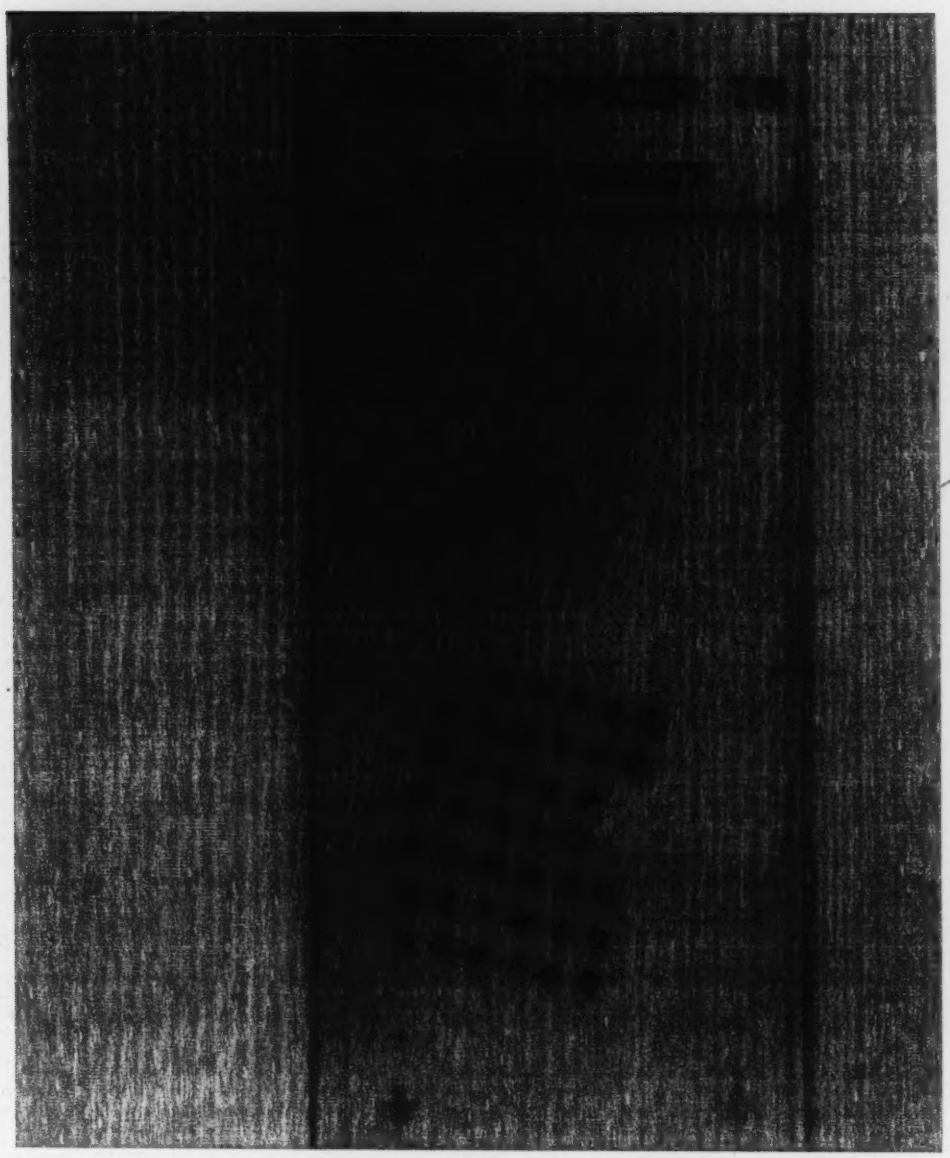

Figure 4.3: Photograph of a gastric cancer multi-tumor tissue array constructed using 1 mm core needle biopsies of archival gastric cancer tissue.

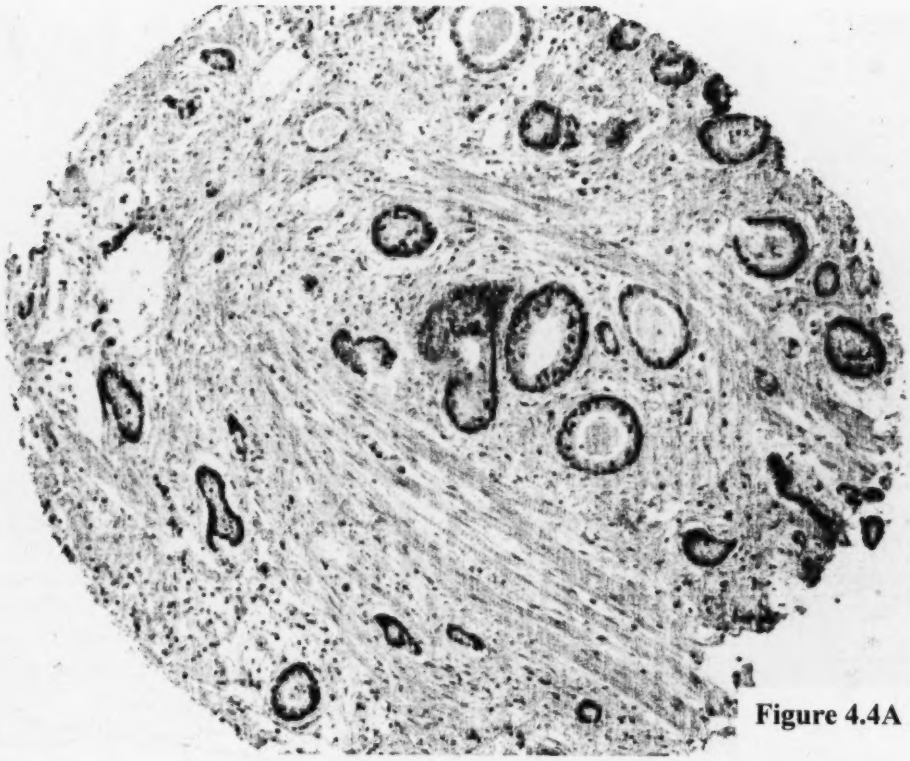

Figure 4.4A: Immunohistochemical staining of COX-2 in a gastric cancer multi-tumor tissue array. (A) Photomicrograph of a 1 mm core biopsy of gastric cancer demonstrating COX-2 negative immunoreactivity (magnification 10 X).

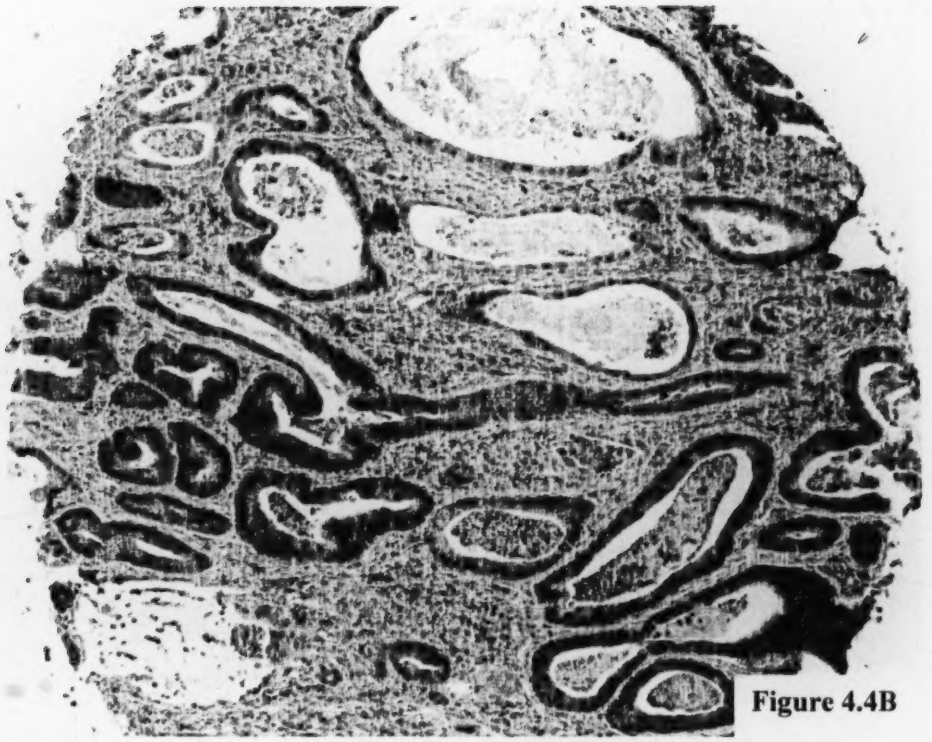

Figure 4.4B

Figure 4.4B: Immunohistochemical staining of COX-2 in a gastric cancer multi-tumor tissue array. (B) Photomicrograph of a 1 mm core biopsy of gastric cancer in which COX-2 immunoreactivity is strongly identified (2+) in the cytoplasm of the cancer cells (magnification 10 X).

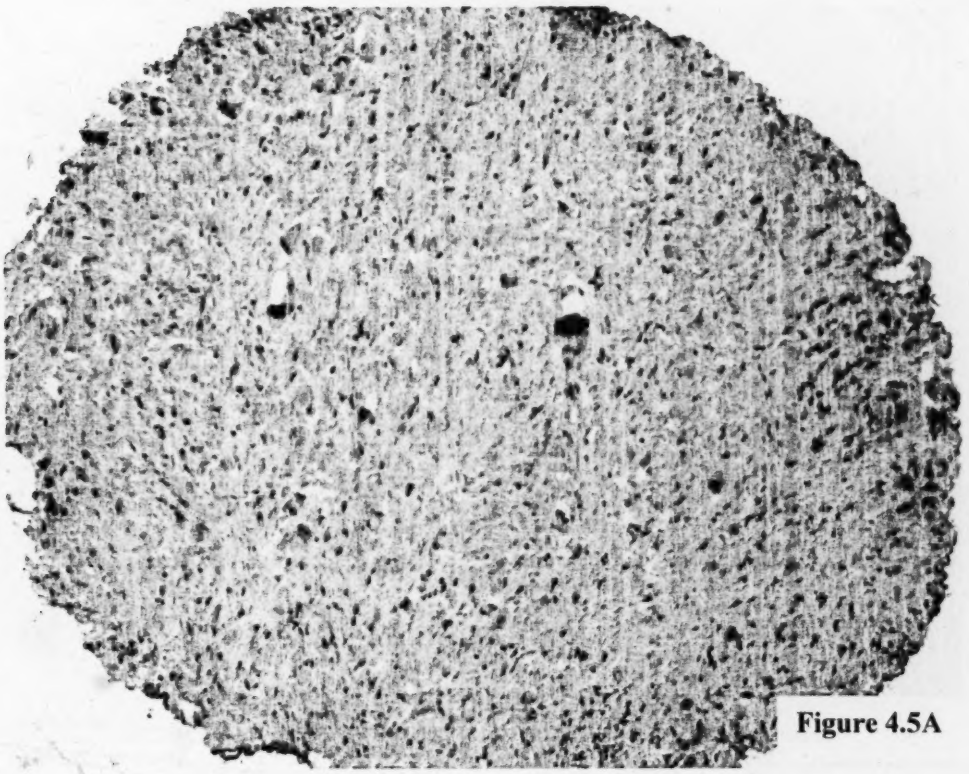

Figure 4.5A

Figure 4.5A: Immunohistochemical staining of MMP-9 in a gastric cancer multi-tumor tissue array. (A) Photomicrograph of a 1 mm core biopsy of gastric cancer demonstrating MMP-9 negative immunostaining (magnification 10 X).

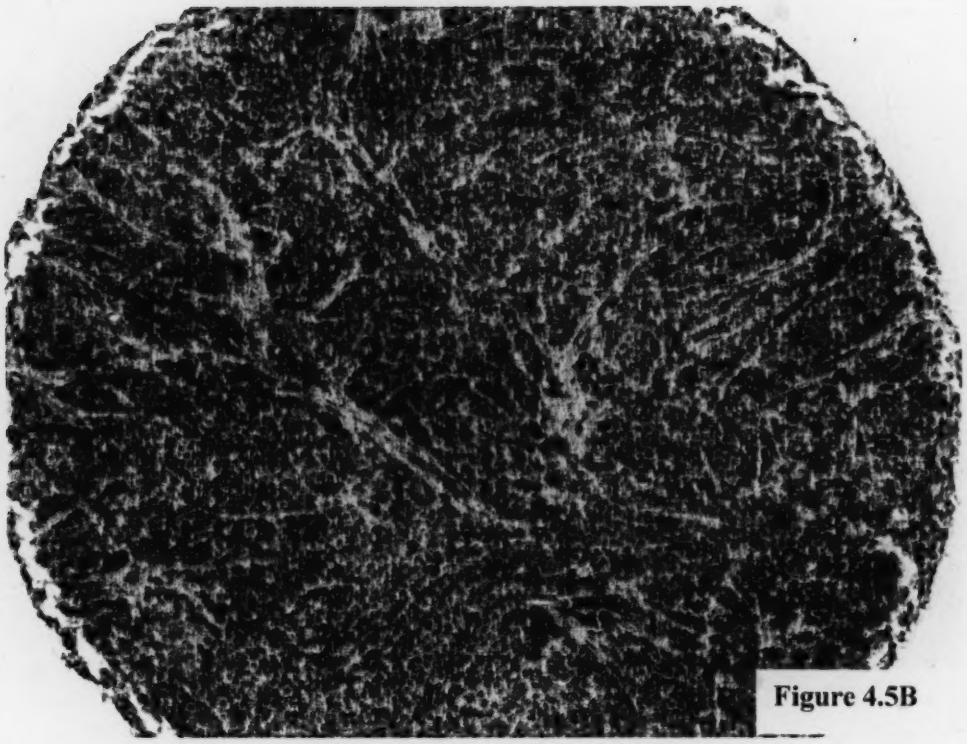

Figure 4.5B: Immunohistochemical staining of MMP-9 in a gastric cancer multi-tumor tissue array. (B) Photomicrograph of a 1 mm core biopsy of gastric cancer in which MMP-9 immunoreactivity is strongly identified (2+) in the cytoplasm of the tumor cells (magnification 10 X).

Chapter Five

5.1 Summary and Conclusions

Population-based studies overcome the issues of selection bias encountered by specialized hospital units. Population-based studies, by including all diagnosed cases, address surgical/pathological and patient heterogeneity, thereby allowing comparisons of survival estimates between geographically defined populations. Clinicopathologic data gathered from population-based cancer cohorts may be used as surrogate markers of tumor behavior to assist in prognostication, treatment planning and provide a platform from which future hypotheses may be generated and tested.

Tumor thickness (T status), lymph node involvement (N status), metastatic disease (M status), tumor histology, residual tumor status and proximal resection margin (esophageal margin) are independent multivariate predictors of long-term survival in a population-based cohort of patients with primary gastric adenocarcinoma. Overall, T status and N status were the most significant predictors of disease-specific survival. In patients with lymph node negative gastric cancer, lymphovascular invasion (LVI) emerges as an independent predictor of survival. The relationship between LVI, T status and ultimate lymph node involvement with cancer is yet to be clearly delineated; however, our studies suggest that LVI is an important surrogate marker of tumor behavior and its presence may suggest biologically aggressive disease.

Despite the utility of surrogate predictors of survival, more specific markers of biologic behavior are needed to improve prognostication and to provide targets for improved management strategies. Multi-tumor tissue arrays allow an efficient and high throughput analysis of hundreds of archival gastric cancer samples simultaneously. Immunoreactivity studies of selected protein markers, based upon proposed protein expression pathways, provide a molecular link to observed clinicopathological predictors of survival. Cyclooxygenase-2 (COX-2) immunoreactivity is significantly associated with T status, lymph node involvement and tumor grade. COX-2 was significantly associated with matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) immunoreactivity. Overall, COX-2 may be involved in the early process of tumor invasion by facilitating the activation of MMP-9 and MMP-2 by enhancing tumor access to the lymphovascular space through degradation of the base membrane.

DNA microarray technology is a rapidly growing field of functional molecular biology, providing insight into the gene-expression profiles which may govern tumor behavior. In a preliminary study we found a differential expression of oligophrenin-1 in the LVI positive group relative to the LVI negative group. Oligophrenin-1 may be involved in the activation and mediation of cellular migration and invasion through MMP-9. Activation of MMP-9 may be coordinated with COX-2 through a common molecular pathway mediated by oligophrenin-1.

Ribophorin-II, a protein complex associated with T-cell activation, was differentially expressed in the LVI-positive group relative to the LVI-negative group. Ribophorin-II may be suppressed in tumors with aggressive biologic behavior, giving tumor cells a survival advantage once the lymphovascular spaces have been breached.

5.2 Agreement of the Major Findings with the Literature

Consistency of the population-based data with results reported in the literature adds validity to a study's findings. The clinicopathologic predictors of survival and disease-specific survival estimates reported in this thesis are consistent with results from similar population-based studies.[192-195] The role of LVI has previously been suggested to have prognostic significance when examined in lymph node negative gastric cancer patients.[112, 113] There are no published studies that have specifically investigated the relationship between LVI and immunoreactivity profiles of COX-2, MMP-2 and MMP-9. Furthermore, there are no published studies which have attempted to identify a gene-expression profile predictive of LVI.

5.3 Limitations

There are several potential limitations to this study. First, although the Alberta Cancer Registry is a provincial database regulated by law, incomplete data entry, coding inaccuracies, missing data and changes in coding nomenclature are potential sources of error that may introduce bias in to a retrospective study. Second, the validity of retrospective cohort studies may be limited by the presence of unknown or immeasurable confounding factors.

With respect to the prospective component of this study, the greatest limitation associated with the collection and banking of fresh gastric cancer was the accrual of sufficient patients meeting the inclusion and exclusion criteria to provide adequate power

to the study. Despite capture of most patients presenting with gastric cancer, several patients were found to be ineligible for reasons other than unresectable disease including, prolonged ischemia time of the excised tumor, failure to provide consent, alternate diagnosis following pathologic assessment, laboratory error in tumor processing and insufficient RNA isolation from freshly banked tumors. As a consequence of reduced tumor samples, the power to detect significant gene-expression profiles predictive of tumor behavior limited definitive study conclusions. Finally, DNA microarrays were expensive to produce and the analysis required a significant knowledge and expertise to carry out. Nevertheless, the data generated provided information on which future hypotheses may be generated and tested.

Multi-tumor tissue arrays, although highly efficient, were also expensive to generate and were limited by the availability of archival gastric cancer tissue. Despite these limitations, the multi-tumor arrays were a useful technique in examining potential protein markers and correlating these with clinicopathologic predictors of survival.

5.4 Future Directions

The results of this study demonstrated generalizability with similar population-based studies of gastric cancer. The data generated from multivariate analyses offered insight into the behavior of gastric cancer by identifying clinicopathologic predictors of survival. Protein markers were then selected as targets for immunoreactivity in an attempt to further delineate the biologic pathways involved in LVI in gastric cancer. DNA microarray studies were conducted on a small prospective cohort of gastric cancer patients, in an attempt to identify a gene-expression profile predictive of LVI, and to identify potential gene targets for subsequent hypothesis testing.

The collection and tumor banking of fresh gastric cancer specimens coupled with improved microarray analyses using microarray platforms with greater gene densities offers the opportunity to further explore the observations from this study. The collection of a larger cohort of patients would provide the necessary statistical power to the study. Tissue array studies using the protein markers oligophrenin-1 and ribophorin-II identified from the microarray studies may be used to validate the present study's observation and provide a platform for future studies examining the role of immune surveillance in gastric cancer with respect to lymphovascular invasion.

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APPENDIX 1

Japanese Classification for Gastric Carcinoma (JCGC)

LN Group	Anatomic Location	D category
Group 1	left cardiac, right cardiac, greater & lesser curvature Supra-pyloric & infra-pyloric	D1
Group 2	left gastric, common hepatic, splenic artery, splenic hilum Hepatic proper, celiac	D2
Group 3	hepatoduodenal, posterior pancreas, root of mesentery, Paraesophageal, diaphragmatic	D3

LN = lymph node
D = extent of surgical resection according to western nomenclature
D1 = group 1; D2 = groups 1 + 2; D3 = groups 1+ 2 + 3 + para-aortic dissection.
Source: Karpeh MS, et al. Annals of Surgery. 2000; 232(3): 362 – 371).

APPENDIX 2

American Joint Committee on Cancer Classification of Gastric Cancer (5th Ed).

T = Primary Tumor

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Invades lamina propria/submucosa
T2	Invades muscularis propria/subserosa
T3	Penetrates serosa
T4	Invades adjacent structures

N = Lymph Node Status

Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph nodes involved
N1	Metastasis in 1 – 6 regional lymph nodes
N2	Metastasis in 7 – 15 regional lymph nodes
N3	Metastasis in more than 15 regional lymph nodes

M = Distant Metastasis

Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage Grouping

Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T1	N1	M0
	T2	N0	M0
Stage II	T1	N2	M0
	T2	N1	M0
	T3	N0	M0
Stage IIIA	T2	N2	M0
	T3	N1	M0
	T4	N0	M0
Stage IIIB	T3	N2	M0
Stage IV	T4	N1, N2, N3	M0
	T1, T2, T3	N3	M0
	Any T	Any N	M1

Source: http://tnm.uicc.org (With Permission: John Wiley & Sons, Inc.)

APPENDIX 3

Meta-analyses of Randomized Clinical Trials of Adjuvant Chemotherapy

Reference	Patients	RCT	OR	95%CI	p-value
Hermans et al.[85]	2,096	11	0.88	0.78 - 1.08	NS
Earle et al.[86]	1,990	13	0.80	0.66 - 0.97	0.024
Mari et al.[87]	3,658	20	0.82	0.75 - 0.89	<0.001
Janunger et al.[88]	3,962	21	0.84	0.74 - 0.96	n/a
Panzini et al.[89]	3,118	17	0.72	0.62 – 0.84	n/a
Hu et al.[90]	4,543	14	0.56	0.40 – 0.79	<0.001
Janunger et al.[91]	1,928	25	0.94	0.77 – 1.14	NS

Significance is noted by p-value <0.05; NS = Not Significant; n/a = not reported
Patients = number of patients included in meta-analyses
RCT = randomized clinical trial and number of studies included in the meta-analyses.
95% CI = 95% confidence interval

APPENDIX 4

Data Collection and Variable Coding Form

A) Demographic Data

Name (last, first, initial):_____ Code ID:_____

ID (Hosp, #):_____ Cross Cancer ID #:_____

DOB (dd/mm/yy):_____ Date of Dx (dd/mm/yy):_____

Date of Surgey (dd/mm/yy):_____

Hospital (UAH=1, RAH=2, GNH=3, MIS=4):_____

B) Clinicopathologic Data

T status (0 – 4):_____ N status (0 – 3):_____ M status (0 – 1):_____

Lymph nodes resected:_____ Lymph nodes positive:_____

Stage (1 - 4):_____ **Note: Missing variable = 88**
Unresectable tumor = 99

Grade (1 -3):_____

Lymphovascular status (0 – 1):_____

Histology (8140=adenoca, 8144=intest, 8145=diffuse, 8480=mucin, 8490=signet
8020=undiff.):_____

Tumor Size (cm, biopsy only = 77):_____

Margin: Proximal/Esophageal (0 – 1):____

Distal/Duodenal (0 – 1):____

Surgery Type (Total=1, Subtotal=2, Bypass=3, Lap=4, Unresect=99):_____

In-hospital Mortality (0 – 1):_____ Adjuvant Therapy (0 – 1):_____

Survival status (0 – 1):_____ Date of last F/U (dd/mm/yy):_____

Date of Death (dd/mm/yy):_____

APPENDIX 5

Clinicopathologic Data Coding Form - List of Variables on the Working File

1. acb_no	unique identifier	
2. dob	Date of Birth	
3. sex	Gender	
	0	female
	1	male
4. surgdate	Date of surgery	
5. dxdate	Diagnosis date	
6. T	tumor thickness	
	1	lamina propria/submucosa
	2	muscularis propria/subserosa
	3	penetrates serosa
	4	invades adjacent structures
7. N	regional lymph nodes	
	0	no nodes involved
	1	1-6 nodes
	2	7-15 nodes
	3	> 15 nodes
8. M	distant metastasis	
	0	no metastasis
	1	distant metastasis
9. Stage	WHO pathological stage	
	1.1	Ia
	1.2	Ib
	2.0	II
	3.1	IIIa
	3.2	IIIb
	4.0	IV
10. Morph	Tumor histology	
	8140	adenocarcinoma
	8144	intestinal
	8145	diffuse
	8480	mucinous
	8490	signet-cell
	8020	undifferentiated
11. Size	Tumor diameter (cm)	
12. tumorsz	Tumor diameter (cm)	
	77	endoscopic biopsy
13. Lymph	Lymphovascular invasion	
	0	no
	1	yes
	99	Missing

14. Grade	Tumor differentiation
	1 well
	2 moderate
	3 poor
15. Esoph	Esophageal margin status
	0 negative
	1 positive
16. Duo	Duodenal margin status
	0 negative
	1 positive
17. Surgtyp	Type of surgical resection
	1 total gastrectomy
	2 subtotal gastrectomy
	3 surgical bypass
	4 laparotomy only
	99 unresectable
18. year	Year of surgical therapy
	1991 - 1997
19. di_hosp	In-hospital mortality
	0 no
	1 yes
20. chemo	Adjuvant chemotherapy (type and cycles)
	0 not applicable
	1 5FU alone or in combination
21. rads	Adjuvant radiotherapy (total Gy)
22. status	Survival status
	0 dead
	1 alive
23. deaddate	Date of death
24. followup	Date of last follow-up (if alive)
25. lossfu	Lost to follow-up
	0 no
	1 yes
26. agegrp	Age group (yrs)
	1 25-50
	2 50.1-60
	3 60.1-70
	4 > 70.1
27. noderec	Number of lymph nodes resected
28. nodpos	Number of lymph nodes positive

APPENDIX 6

Peripheral Blood Sample Requisition Form (UAH)

PHN / Healthcare Number | Accession # | **Research Requisition** | 14-Dec-01

LABORATORY MEDICINE AND PATHOLOGY
Client Response Centre (780) 407-7484
CAPITAL HEALTH REGION LABORATORIES
DYNACARE KASPER MEDICAL LABORATORIES

☐ M ☐ F | Patient Legal Name (Last) | (First) | (Initial) | DOB DD MM YY

Address | City | Prov. | Postal Code

Chart # | Patient Phone # | Lab #

☐ Copy to
Name
Physician Code
Address

Ordering Physician / Practitioner | Physician Code

Ordering Address / Location | Report Location Code

Report address if different

Specimen Event Type
IP ☐ IN PT
OP ☐ OUT PT
AP ☐ AMBUL
HC ☐ HMCARE
ST ☐ STAFF
EN ☐ ENVIRON
WCB ☐ WORKER'S COMP

Call 407-6615 with any questions regarding requisition or specimen handling.

Research Bill Acct # ACB

Date specimen collected DD MM YY | Col. Location | Collector

TIME (24 h)

Fasting # of hours ____
☐ Microcollection

SPECIMEN TYPE
Blood ☐ serum ☐ plasma ☐ whole blood ☐ microcollection
Urine / Feces ☐ Random ☐ 24 h ☐ Timed, other
Total volume
Start time / date
Stop time / date
Other

DIAGNOSIS

LAB CONTROL NUMBER
RES1118
ACB PolyomX Study

COLLECTION PROTOCOL

- **Buffy Coat:** Collect three 5 mL EDTA/mauve top tubes.
- **Serum:** Collect one 10 mL plain (no gel) red top tube.

Processed by_____(*Tech ID*)

HANDLING AND STORAGE PROTOCOL

Aliquot tubes and labels are located in the RES1118 research tray in the Clinical Trials Lab.

Buffy Coat:
Allow specimens to sit at room temperature for 30 minutes. Spin specimens for 15 minutes at 3500 rpm. Take the spun tubes and two aliquot tubes to the hematology technologist for aliquotting.

Hematology Technologist:
Using a pipette carefully transfer 200 µL of buffy coat from the first mauve top to one of the two cryovials provided. Label tube with Buffy #1 label provided. Then transfer the buffy coats from both the second and third mauve tops to the remaining cryovial. Label tube with Buffy #2 label provided. Return the specimens to specimen control for freezing.
Store frozen at -70°C.

Serum:
Spin specimen for 15 minutes at 3500 rpm. Make as many 1.5 mL aliquots of serum as possible. Label tubes with patient's initials and their PHN.
Store frozen at -70°C.

Contact: Kathryn Calder, 432-8803

ACCESSION PROTOCOL AND TEST REQUESTS

Clinical Trials Staff:
Photocopy research requisition and give the copy to the CCI staff when they pick up the specimens. No shipping required.

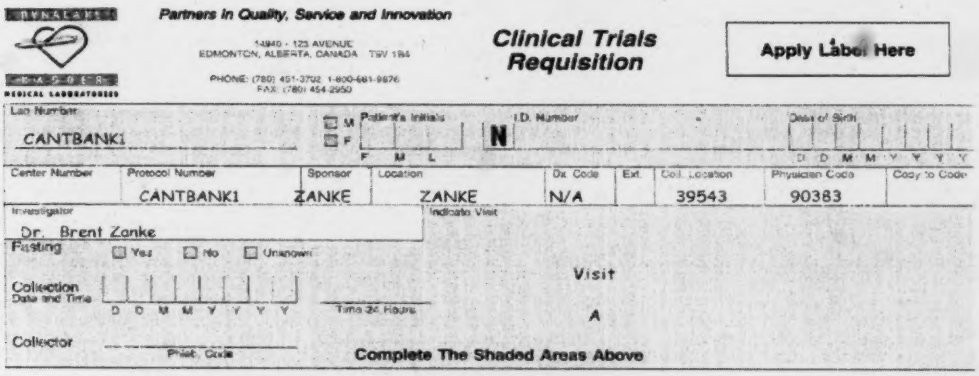
Specimen for Research Send-out Only

UAH DOES NOT ENTER OR ANALYSE

SP.8255 Feb 2001

APPENDIX 7

Peripheral Blood Sample Requisition Form (DKML)



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PHONE: (780) 451-3702 1-800-661-9876
FAX: (780) 454-2950

DASER
MEDICAL LABORATORIES

Clinical Trials Requisition

Apply Label Here

Lab Number: CANTBANK1

Patient's Initials: ☐ M ☐ F — F M L

I.D. Number: N

Date of Birth: D D M M Y Y Y Y

Center Number	Protocol Number	Sponsor	Location	Dx. Code	Ext.	Coll. Location	Physician Code	Copy to Code
	CANTBANK1	ZANKE	ZANKE	N/A		39543	90383	

Investigator: Dr. Brent Zanke

Indicate Visit

Fasting: ☐ Yes ☐ No ☐ Unknown

Collection Date and Time: D D M M Y Y Y Y — Time 24 Hours

Visit: A

Collector — Phlet. Code

Complete The Shaded Areas Above

RESEARCH STAFF

- **Complete requisition with patient information and visit status.**
- **Send this requisition with the patient to the lab.**

LAB STAFF

- **Indicate the date and time of collection on requisition.**
- **Collect two 7mL EDTA tube, mix well and allow to sit for 30 min. Centrifuge at 3500 rmp for 15 min. Remove buffy coat place 200uL into one of the cryovials, place the remainder into another one of the cryovials provided. Label tubes with patient initials and ID using felt tip pen. Place into biohazard bag freeze at -70.**
- **Collect one 10mL plain red top, allow to clot for 30 min. Centrifuge and aliquot serum into as many full cryovials as possible. Label tubes with patient initials and ID using felt tip pen. Place into same biohazard bag as buffy coat (one bag per patient) freeze at -70.**
- **Ensure initials and ID numbers are on the labels provided along with a copy of this requisition. Place in side pocket of the biohazard bag.**
- **Research staff will retrieve tubes weekly for analysis.**
- **Send the DKML requisition through your regular DKML courier ATTENTION "Clinical Trials".**

DATA ENTRY STAFF

- **Log in for Central Lab Collection** **JHAN**

APPENDIX 8
Health Ethics Approval & Update: University of Alberta

Health Research Ethics Board biomedical research health research
2J2.27 Walter Mackenzie Centre
University of Alberta, Edmonton, Alberta T6G 2R7
p.780.492.9724 f.780.492.7303
ethics@med.ualberta.ca
3-48 Corbett Hall, University of Alberta
Edmonton, Alberta T6G 2G4
p.780.492.0839 f.780.492.1626
ethics@www.rehabmed.ualberta.ca

ETHICS APPROVAL FORM

Date: November 2002

Name(s) of Principal Investigator(s): Dr. Stewart Hamilton

Department: Surgery

Title: Global gene-expression and single nucleotide polymorphism analysis of gastric cancer: A novel approach to classification, treatment planning and prognosis

Protocol:

The Health Research Ethics Board (Biomedical Panel) has reviewed the protocol involved in this project which has been found to be acceptable within the limitations of human experimentation.

The Research Ethics Board assessed all matters required by section 50(1)(a) of the Health Information Act. The REB Panel determined that the research described in the ethics application involves the use of anonymous biological samples normally discarded for which subject consent for access to personally identifiable health information would not be reasonable, feasible or practical. Subject consent therefore is not required for access to the personally identifiable health information described in the ethics application

Signed - Chairman of Health Research Ethics Board (Biomedical)

D. W. Morrish, M.D.
Chairman, Health Research Ethics Board
Biomedical Panel

This approval is valid for one year

Health Research Ethics Board biomedical research health research

2J2.27 Walter Mackenzie Centre
University of Alberta, Edmonton, Alberta T6G 2R7
p.780.492.9724 f.780.492.7303
ethics@med.ualberta.ca

3-48 Corbett Hall, University of Alberta
Edmonton, Alberta T6G 2C4
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ethics@www.rehabmed.ualberta.ca

NOV 2 8 2002

cc Dr. B. Dicken

November 27, 2002

Our file #4430

Dr. S.G. Hamilton
Department of Surgery
2D2 WMC

Dear Dr. Hamilton:

Re: Global gene expression and single nucleotide polymorphism analysis of gastric cancer: A novel approach to classification, treatment planning and prognosis.

Thanks to you and Dr. Dicken for submitting the above study to the Research Ethics Board. Dr. Morrish has reviewed your application to conduct this research on biological specimens which are normally discarded, and has approved it on behalf of the committee. Your approval form is enclosed. In order to comply with the Health Information Act, a copy of the approval form is being sent to the Office of the Information and Privacy Commissioner.

Next year, a few weeks prior to the expiration of your approval, a Progress Report will be sent to you for completion. If there have been no major changes in the protocol, your approval will be renewed for another year. All protocols may be subject to re-evaluation after three years.

For studies where investigators must obtain informed consent, it is a policy of the Faculty of Medicine & Dentistry that signed copies of the consent form must be retained, and be available on request. They should be kept for the duration of the project and for a full calendar year following its completion.

Approval by the Health Research Ethics Board does not encompass authorization to access the patients, staff or resources of the Capital Health Authority or other local health care institutions for the purposes of research. Enquiries regarding institutional approval requirements should be directed to C. Jaster, CHA Regional Research Administration office, #1800 College Plaza, phone 407-1372.

Yours sincerely,

Judith

Judith R. Abbott (Ms.)
Administrative Coordinator
Health Research Ethics Board (Biomedical Panel)

/ja
enc.

APPENDIX 9

Health Ethics Approval and Update – Cross Cancer Institute

Alberta Cancer Board

Standard Life Centre
#1220, 10405 Jasper Avenue
Edmonton, AB T5J 3N4
Canada

Phone: (780) 412-6300
Fax: (780) 412-6326

SERVING ALBERTANS THROUGH THE FOLLOWING FACILITIES:

Cross Cancer Institute (Edmonton)

and its associate cancer centres:
Central Alberta Cancer Centre (Red Deer)
Grande Prairie Cancer Centre

and its community cancer centres:
Aspen (Barrhead)
Bonnyville
Camrose
Northern Lights Regional Health Services (Fort McMurray)
Hinton
Peace (Peace River)

Tom Baker Cancer Centre (Calgary)

and its associate cancer centres:
Lethbridge Cancer Clinic
Medicine Hat Cancer Clinic

and its community cancer centres:
Headwaters (High River)
Regional Health Authority #5 (Drumheller)

Southern Alberta Cancer Research Centre (Calgary)

Screen Test:
The Alberta Program for the Early Detection of Breast Cancer with clinics in Calgary and Edmonton and mobile units serving rural Alberta

11094

10 October 2002

Dr. Carol Cass
Department of Experimental Oncology
Cross Cancer Institute

Dear: Dr. Cass

RE: ETH-02-87-30: Global Gene-expression and Single Nucleotide Polymorphisms Analysis of Gastric Cancer: A Novel Approach to Classification, Treatment Planning and Prognosis

Thank you for Bryan Dicken's presentation of the above-mentioned study at our meeting on 10 October 2002. I am pleased to grant approval to your participation in the above noted study on behalf of the Research Ethics Board (REB).

Please note that this approval is subject to the following conditions:

- please submit your approved Clinical Priorities Assessment form from the Cross Cancer Institute before initiating the study;
- an agreement between the Sponsor and the Alberta Cancer Board governing conduct of the study must be executed before commencing the study (if applicable);
- if there are any other changes to the protocol during the year, or if any serious adverse events to the treatment are found, a letter describing the changes/reactions must be forwarded to the REB as per the Cross Cancer Institute Policy 10A.16;
- an Annual Renewal form must be submitted two months prior to the deadline date of **07 October 2003** (one year from date of the convened REB meeting), containing the information as per our annual renewal form;
- a Final Report must be submitted at the termination of the project.

The deliberations of the REB included all elements described in Section 50 of the Health Information Act, and found the study to be in compliance with all the applicable requirements of the Act. Access to personal identifiable health information from the Cancer Registry has been approved at Alberta Cancer Board Consent is not required from these subjects as it is a chart review.

The Alberta Cancer Board REB, complies with the following guidelines and regulations:

- Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans;
- Health Information Act which has been proclaimed on April 25, 2001 in Alberta;
- Health Canada, as defined in C.05 (Part C Division 5) (1024 - Clinical Trials) of the Food And Drug Regulations -Amendment and the Therapeutic Products Directorate Guidelines /ICH Harmonized Tripartite Guidelines - Good Clinical Practice: Consolidate Guidelines;
  National Institutes of Health - Code of Federal Regulations (USA); and

Alberta Cancer Board

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SERVING ALBERTANS THROUGH THE FOLLOWING FACILITIES:

Cross Cancer Institute (Edmonton)

and its associate cancer centres:
Central Alberta Cancer Centre (Red Deer)
Grande Prairie Cancer Centre

and its community cancer centres:
Aspen (Barrhead)
Bonnyville
Camrose
Northern Lights Regional Health Services (Fort McMurray)
Hinton
Peace (Peace River)

Tom Baker Cancer Centre (Calgary)

and its associate cancer centres:
Lethbridge Cancer Clinic
Medicine Hat Cancer Clinic

and its community cancer centres:
Headwaters (High River)
Regional Health Authority #5 (Drumheller)

Southern Alberta Cancer Research Centre (Calgary)

Screen Test:
The Alberta Program for the Early Detection of Breast Cancer with clinics in Calgary and Edmonton and mobile units serving rural Alberta

11094

- our institution has been approved by the Office for Human Research Protections in the United States.

Members of the REB who are named as investigators or co-investigators in research studies do not participate in discussion related to, nor vote on, such studies when they are presented to the REB.

Please accept the Board's best wishes for success in your research.

Sincerely,

Sunil J. Desai, M.D.
Chair, Research Ethics Board

PC:
Kathryn Nimchuk (PCRP)
CPA
Brenda Bird-Cantelon
OIPC

RECEIVED
SEP 16 2003

Alberta Cancer Board

ALBERTA CANCER BOARD

Provincial Office
1220, Standard Life Building
10405 Jasper Avenue
Edmonton, Alberta
Canada T5J 3N4
Tel: (780) 412-6300

ACB Provincial Office
Edmonton

Tertiary Cancer Centres
Cross Cancer Institute
Tom Baker Cancer Centre

Associate Cancer Centres
Central Alberta Cancer Centre
(Red Deer)
Grande Prairie Cancer Centre
Lethbridge Cancer Centre
Medicine Hat Cancer Centre

Community Cancer Centres
Barrhead
Bonnyville
Camrose
Canmore
Drayton Valley
Drumheller
Ft. McMurray
High River
Hinton
Lloydminster
Peace River

Epidemiology, Prevention & Screening
Calgary

Medical Affairs & Community Oncology
Edmonton

Research
Edmonton

Alberta Cancer Foundation
Accepts donations in support of ACB facilities and programs
Toll-free: 1-866-412-4222

www.cancerboard.ab.ca

September 11, 2003

File #: 17126
ETH-02-87-30

Carol E. Cass
Oncology
Cross Cancer Institute

Dear Dr. Cass:

RE: Global Gene-expression and Single Nucleotide Polymorphisms Analysis of Gastric Cancer: A Novel Approach to Classification, Treatment Planning and Prognosis

The Research Ethics Board (REB, Full Board) at its meeting on September 09, 2003, reviewed the annual progress report that you provided on the above-mentioned research study.

I am pleased to advise you that approval for the study has been granted for another year from the date of your last aproval up to and including **October 07, 2004**.

If there are any changes to the protocol or consent form during the year, or if any adverse reactions to the treatment are found, the REB requests that you forward a letter describing the changes/reactions, together with an updated consent form, to the Research Administration Office.

Your next annual progress report for this study should be submitted two months prior to the above deadline date. Failure to do so will jeopardize continuation of the study and accrual of patients beyond the deadline date.

Please accept the Board's best wishes for continued success in your research

Sincerely,

Sunil J. Desai, M.D.
Chair, Research Ethics Board

PC: Paula Langenhoff, Clinical Research Office
Roseanne Gallant, OIPC

ALBERTA CANCER BOARD
RESEARCH ETHICS COMMITTEE (REC)

Annual renewal/amendment form for approved studies: PLEASE TYPE (add extra sheets as necessary)

Principal Investigator: Dr. Carol E. Cass Project: ETH-02-87-30

Names of all co-investigators: Dr. Chris J. de Gara

Protocol Title: Global Gene-expression and Single Nucleotide Polymorphisms Analysis of Gastric Cancer: A Novel Appro
Classification, Treatment Planning and Prognosis

Date of original approval: 10/10/02 Renewal Number:

Reporting period: October 7, 2003

Status of protocol/study: continuing [✓] closed to accrual [complete [ ] abandoned [ ]
If abandoned, why?

Total number of subjects required for study:

Number of subjects accrued to date (locally) 48 (total) 50. Anticipated date of completion of accrual

Are accrued subjects still being followed? Yes [✓] No [ ]

Have there been problems recruiting subjects? Yes No [✓]

Have there been any previously unidentified risks or benefits noted: Yes [No [✓]

If yes, please explain:

Have there been any other changes to your protocol (eg study design, changes in method of subject recruitment, funding st
etc) since this study was first approved: Yes [ ] No [✓]
If yes, please explain:

Have any serious events occurred during the reporting period? Yes No [✓]. If yes, list them below and the evaluatic
their significance.

Has this study been site-audited? Yes [ ] No [If yes, date of last audit:
If no, explain why.

Do you consider the approved consent form to be still appropriate? Yes [✓] No [ ]. If no, explain why and submit a new on

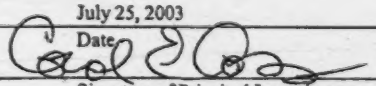
Do you have preliminary results: Yes [ ] No [✓]. If yes, attach copies.

Please attach copies of (1) incident reports, and (2) any other related information on study.

Will there be any publications or presentations? Yes [✓] No [

Summarize the research progress achieved over the last year: Accural of gastric tissue with enough samples to allow microar
analysis and correlation studies with clinicopathologic variable
If now complete, what has this project led to? Presently the study is progressing very well and we anticipate results in the nea
future

Brian Dicken — July 25, 2003
Name of Person Completing Report — Date

Carol E. Cass — 
Name of Principal Investigator — Signature of Principal Investigator

Names of others requiring copies of approval letter

ALBERTA CANCER BOARD

CROSS CANCER INSTITUTE

MEMORANDUM

TO: **Dr. Michael Smylie**
c/o Kathryn Nimchuk

DATE: July 4, 2002

Name of Applicant:
Dr. Bryan Dicken

Affiliation and Address of Applicant:
General Surgery Resident University of Alberta and CIP Postgraduate Research Student
Address: CCI, Department of Surgery, Room 2004

Study Name (if applicable):
Gastric Cancer and Genetic Profiling

Purpose of Visit:
To correlate registry data that we have already analyzed

Photocopying required: Yes ☑ No ☐ *After Hours Access:* Yes ☑ No ☐
Justification for above request(s)
Bryan is a resident and may need to do work after hours. He will need to do some photocopying for completion of patient demographics and pathology reports.

Start and End Dates of Visit:
July 1, 2002 – January 31, 2003

I, as the recipient of the Director's Approval authorizing my access to patient information, hereby agree to maintain and protect the confidentiality of the information acquired for the purpose(s) specified above. Further, I hereby release the Alberta Cancer Board, its members, officers and employees from any claims, which may arise as the result of the unauthorized release of this information.

Signature

Dr. C. de Gara
CCI Investigator/Supervisor Name

Signature

Approval Granted by: Michael Smylie
~~Brent Zanke, Director~~
Dr. Michael Smylie

Date

HEALTH RECORDS USE			
ACCESS to PATIENT CREATED Monitor Institution	Monitor ID	Date	Created By
ACCESS to PATIENT REMOVED		Date	Removed By
PASSWORD REMOVED		Date	Removed By

ACTI 750-120\Facility Coord\Frm\Study Approval

October 2001

ALBERTA CANCER BOARD

CROSS CANCER INSTITUTE

MEMORANDUM

TO: **Dr. Carol Cass**
Director, CCI (Acting)

DATE: May 13, 2003

Name of Applicant: **Dr. Bryan Dicken**

Affiliation and Address of Applicant: **General Surgery Resident, University of Alberta and CIP Postgraduate Research Student**
CCI Address: **Department of Surgery, Room 2004**

Study Name (if applicable): **Global Gene-Expression and Single Nucleotide Polymorphisms Analysis of Gastric Cancer: A Novel Approach to Classification, Treatment Planning, and Prognosis**

Purpose of Visit: **To review records and collect data as specified in the above mentioned protocol (ACB Ethics approval number ETH-02-87-30).**

Photocopying required: Yes X No ☐ *After Hours Access*: Yes X No ☐

Justification for above request(s) **Other clinical commitments may necessitate that Dr. Dicken may have to review records after regular office hours. Some photocopying will be necessary for completion of patient demographics and pathology reports.**

Start and End Dates of Visit: **May 1, 2003 – May 1, 2004 (This is a renewal of existing privileges.)**

I, as the recipient of the Director's Approval authorizing my access to patient information, hereby agree to maintain and protect the confidentiality of the information acquired for the purpose(s) specified above. Further, I hereby release the Alberta Cancer Board, its members, officers and employees from any claims which may arise as the result of the unauthorized release of this information.

Signature

C. de Gara — CCI Investigator/Supervisor Name

C. de Gara — Signature

Approval Granted by: ~~Carol E. Cass, Director (Acting)~~ J M. Turc, President & CEO. — May 22 2003 — Date

HEALTH RECORDS USE			
ACCESS to PATIENT CREATED Monitor Institution	Monitor ID	Date	Created By
ACCESS to PATIENT REMOVED		Date	Removed By
PASSWORD REMOVED		Date	Removed By

APPENDIX 10

Gastric Cancer Tissue Collection Consent Form

UNIVERSITY OF ALBERTA
Alberta Cancer Board PolyomX
Dr. Carol Cass, Chair
Dr. John Mackey, Clinical Chair
11560 University Avenue
Edmonton, AB T6G 1Z2
Phone: (780) 432-8771 Fax: (780) 432-8888

Study Title: Alberta Cancer Board PolyomX / Cancer Tissue Banking Study

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carol.cass@

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andrewbe@

Medical Physics Director
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Palliative Care Medicine Director
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Fax: 491-5880
rfainsinger@cha.ab.ca

Radiation Oncology Director
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Fax: 432-8380
robertpe@

Surgical Oncology Acting Director
Dr. Christopher de Gara
432-8337
Fax: 432-8333
chrisdeg@

About Using Tissue for Research

You are going to have a biopsy or surgery to see if you have cancer. Your doctor wil remove some body tissue to do some tests. The results of these tests will be given to you by your doctor and will be used to plan your care.

We would like to keep a tube of your blood and the leftover tissue for future research If you agree, this tissue and blood sample will be kept and used to learn more about cance and other diseases. The tissue will only be used by researchers whose projects were reviewed and approved by the Alberta Cancer Board (ACB) PolyomX managemen committee. Any research done on the tissue must also be approved by an institutiona Research Ethics Board. Please read the question and answer sheet called "How is Tissue Used for Research" to learn more about tissue research.

Your tissue may be helpful for research whether you do or do not have cancer. The research that may be done with your tissue probably will not help you. It might help people who have cancer and other diseases in the future.

Reports about research done with your tissue will not be given to you or your doctor. These reports will not be put in your health record. The research will not have an effect on you care.

Things to Think About

The choice to let us keep the left over tissue for future research is up to you. **No matter what you decide to do, it will not affect your care.** You may still take part in othe treatment trials.

If you decide now that your tissue can be kept for research, you can change your mind a any time. Just contact your study doctor and let him or her know that you do not want us to use your tissue. Then the tissue will no longer be used for research.

In the future, people who do research with tissue may need to know more about your health. This will require the ACB PolyomX to give them reports about your health. This information will not contain your name, address, phone number or any other persona information.

Sometimes tissue is used for genetic research (about diseases that are passed on in families). Even if your tissue is used for this kind of research, the results will not be put in your health records.

Your tissue will be used only for research and will not be sold. You will not be paid for allowing your leftover tissue to be used in research even though the research done with your tissue may help to develop new products in the future. Similarly there will be no cost to you for any tissue collected and stored.

Benefits
The benefits of research using tissue include learning more about what causes cancer and other diseases, how to prevent them, how to treat them, and how to cure them.

Risks
There are very few risks to you. The greatest risk is the release of information from your health records. If the ACB PolyomX program does give researchers any reports about your health, this information will not contain your name, address, phone number or any other personal information.

Making Your Choice
Please read each sentence below and think about your choice. After reading each sentence, check "Yes" or "No." **No matter what you decide to do, it will not affect your care**.. If you have any questions, please talk to your doctor or Kathryn Calder (clinical research nurse) at the Cross Cancer Institute at 780-432-8803. Should you wish to speak to someone not involved in the study about your rights as a study participant, you may contact Camille Wolfe at 780 450-7501, or the Patient Representative at the Cross Cancer Institute at 780-432-8585.

1. My tissue and blood may be kept for use in research to learn about, prevent, treat, or cure cancer.

Yes ☐ **No** ☐

2. My tissue and blood may be kept for use in research to learn about other health problems such as diabetes, Alzheimer's disease, and heart disease).

Yes ☐ **No** ☐

3. My doctor (or someone from ACB PolyomX) may contact me in the future to ask me to take part in more research.

Yes ☐ **No** ☐

UNIVERSITY OF ALBERTA

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robertpe@

Surgical Oncology Acting Director
Dr. Christopher de Gara
432-8337
Fax: 432-8333
chrisdg@

Study Title: Alberta Cancer Board PolyomX

Part 1: Researcher Information

Name of Chair: **Dr. Carol Cass**
Affiliation: Chair, Department of Oncology, University of Alberta
Associate Director of Research, Acting Director, Cross Cancer Institute
Name of Clinical Principal Investigator: **Dr. John Mackey**
Affiliation: Associate Professor, Department of Oncology, University of Alberta
Contact Information: phone 780 432-8221

Part 2: Consent of Subject

	YES	NO
Do you understand that you have been asked to be in a research study?		
Have you read and received a copy of the attached consent form?		
Do you understand the benefits and risks involved in taking part in this research study?		
Have you had an opportunity to ask questions and discuss the study?		
Do you understand that you are free to refuse to participate or withdraw from the study at any time? You do not have to give a reason and it will not affect your care.		
Has the issue of confidentiality been explained to you? Do you understand who will have your records/information?		

Part 3: Signatures

This study was explained to me by: _____
Date: _____

I agree to take part in this study.
Signature of Research Participant: _____
Printed Name: _____
Witness (if available): _____
Printed Name: _____

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.
Researcher: _____

Printed Name: _____

* A copy of this consent form must be given to the participant.

UNIVERSITY OF ALBERTA

How is Tissue Used for Research?

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Administrative Officer
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Medical Physics
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rfainsinger@cha.ab.ca

Radiation Oncology
Director
Dr. Robert Pearcey
432-8749
Fax: 432-8380
robertpe@

Surgical Oncology
Acting Director
Dr. Christopher de Gara
432-8337
Fax: 432-8333
christdg@

Where does tissue come from?

After a person has had a biopsy (or surgery) and all tests have been done, there may be some left over tissue. Sometimes, this tissue is thrown away because it is not needed for the patient's care. Instead, a patient can chose to have the tissue kept for future research. People who are trained to handle tissue and protect donors' rights make sure that the highest standards of quality control are followed by the Alberta Cancer Board (ACB). Your doctor has agreed to help collect tissue from many patients. If you agree, only left over tissue will be saved for research. Your doctor will not take more tissue during surgery than needed for your care.

Why do people do research with tissue?

Research with tissue can help to find out more about what causes cancer, how to prevent it, how to treat it, and how to cure it. Research using tissue can also answer other health questions. Some of these include finding the causes of diabetes and heart disease, or finding genetic links to Alzheimer's.

What type of research will be done with my tissue?

Many different kinds of studies use tissue. Some researchers may develop new tests to find diseases. Others may develop new ways to treat or even cure diseases. In the future, some of the research may help to develop new products, such as tests and drugs. Some research looks at diseases that are passed on in families (called genetic research). Research done with your tissue may look for genetic causes and signs of disease.

How do researchers get the tissue?

Researchers from universities, hospitals, and other health organizations conduct research using tissue. They contact the ACB and request samples for their studies. The ACB reviews the way that these studies will be done, and decides if any of the samples can be used. The ACB gets the tissue and information about you from your hospital, and sends the tissue samples and some information about you to the researcher. The ACB will not send your name, address, phone number, or any other identifying information to the researcher.